Western Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

8-13-2014 12:00 AM

The Implications of Altered Cholinergic Signaling in Cardiac Health and Disease

Ashbeel Roy
The University of Western Ontario

Supervisor
Dr. Marco A. M. Prado
The University of Western Ontario Joint Supervisor
Dr. Robert Gros
The University of Western Ontario

Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
© Ashbeel Roy 2014

Follow this and additional works at: https://ir.lib.uwo.ca/etd

 Part of the Systems and Integrative Physiology Commons

Recommended Citation

Roy, Ashbeel, "The Implications of Altered Cholinergic Signaling in Cardiac Health and Disease" (2014). *Electronic Thesis and Dissertation Repository*. 2337.
https://ir.lib.uwo.ca/etd/2337

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.

THE IMPLICATIONS OF ALTERED CHOLINERGIC SIGNALING IN CARDIAC HEALTH AND DISEASE

(Thesis format: Integrated Article)

by

Ashbeel Roy

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Ashbeel Roy 2014

Abstract

Cardiac remodeling and dysfunction occur prior to the onset of heart failure. Altered regulation of cardiac function by the autonomic nervous system has been implicated in the progression of heart disease. Both altered sympathetic and parasympathetic tone contribute to cardiac disease; however, the role of the parasympathetic nervous system, and specifically acetylcholine (ACh), in cardiac dysfunction has not been fully elucidated. In these studies, we sought to determine whether changes in neuronal and/or non-neuronal ACh release regulate cardiac activity and alter the progression of cardiac remodeling and dysfunction. A systemic decrease in the expression of the vesicular acetylcholine transporter (VAChT), the protein responsible for packaging ACh, led to the development of significant ventricular dysfunction coupled with significant transcriptional changes in cardiac tissue. Furthermore, we identified that murine cardiomyocytes possess an intrinsic cholinergic system, which prevents hypertrophy and molecular remodeling in cardiomyocytes in response to hyperadrenergic stimulation, *in vitro*. In addition, this cardiac non-neuronal cholinergic system (NNCS) is also critical in regulating heart activity and remodeling, *in vivo*. Inhibition of cardiomyocyte-specific ACh secretion led to delayed heart rate recovery following physiological stress, including exercise, as well as significant ventricular remodeling. Cardiomyocytes lacking the intrinsic cholinergic system displayed hypertrophy and molecular remodeling. This NNCS also plays a significant role under pathological conditions as chronic treatment with angiotensin II led to enhanced cardiac remodeling and ventricular dysfunction in mice lacking the NNCS. Additionally, this intrinsic cholinergic system in the heart is also present in human cardiomyocytes, suggesting the conserved expression of prototypic markers of the cholinergic system in man. This system might be of functional significance in cardiac disease as failing human cardiomyocytes exhibit increased VAChT expression, which likely leads to an increase in ACh secretion directly from cardiomyocytes. The increase in VAChT expression may play a protective role in heart failure, as overexpression of VAChT in mice did not reveal adverse phenotypes under physiological conditions. Our data suggest that both neuronal and non-neuronal ACh are

critical in maintaining cardiac homeostasis and deficient cholinergic signaling contributes to ventricular remodeling and cardiac dysfunction.

Keywords

Acetylcholine

Cholinergic signaling

Vesicular acetylcholine transporter

Choline acetyltransferase

Ventricular remodeling

Heart failure

Co-Authorship Statement

The data presented in Chapter 2 of this thesis was previously published in PLoS One on June 29, 2012 under 7(6):e39997. This peer-reviewed research article is titled "An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with reduced cholinergic neurotransmission." All of the experiments in Chapter 2, except Figure 2.3, were performed by Ashbeel Roy under the supervision of Dr. Marco A. M. Prado and Dr. Robert Gros. The MitoSOX experiment to analyze oxidative stress was performed by Diogo Guimarães under the supervision of Dr. Marcus Vinicius Gomes and Dr. Silvia Guatimosim (UFMG, Belo Horizonte, Brazil).

Chapter 3 of this thesis was previously published in the Journal of Molecular and Cellular Cardiology in August, 2012 under 53(2):206-16. The peer-reviewed research article is titled "Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals." The experiments presented in Figures 3.1 and 3.4 were performed by Ashbeel Roy under the supervision of Dr. Marco A. M. Prado and Dr. Robert Gros. All other experiments presented in Chapter 3 were performed in the Guatimosim Lab (UFMG, Belo Horizonte, Brazil) under the direct supervision of Dr. Silvia Guatimosim.

Chapter 4 of this thesis was previously published in the FASEB Journal in December, 2013 under 27(12):5072-82. The peer-reviewed research article is titled "Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart." All of the experiments presented here, except for Figures 4.3, 4.4 and 4.8c, were performed by Ashbeel Roy under the supervision of Dr. Marco A. M. Prado and Dr. Robert Gros. Acetylcholine secretion experiments (Figures 4.3 and 4.4) were performed by William C. Fields under the supervision of Dr. Marco A. M. Prado and Dr. Robert Gros. The siRNA experiment (Figure 4.8c) was performed by Cibele Rocha-Resende and Rodrigo Ribeiro Resende under the supervision of Dr. Silvia Guatimosim (UFMG, Belo Horizonte, Brazil).

All of the experiments presented in Chapter 5, except for Figure 5.4B-E, were performed by Ashbeel Roy under the supervision of Dr. Marco A. M. Prado and Dr. Robert Gros. The M-mode echocardiography experiments (Figure 5.4B-E) were performed by Yin Liu under the supervision of Dr. Qingping Feng (UWO, London, Canada).

All other experiments presented in this thesis were performed by Ashbeel Roy under the supervision of Dr. Marco A. M. Prado and Dr. Robert Gros at the University of Western Ontario in the Department of Physiology and Pharmacology.

Acknowledgments

For wisdom is a defense, and money is a defense: but the excellency of knowledge is, that wisdom giveth life to them that have it.

Ecclesiastes 7:12

Firstly, I would like to extend the greatest of thanks to my supervisor, Dr. Marco Prado, for everything he has done over the past five years. Without your continuing support and guidance, this would not have been possible. In my mind, you are one of the most talented and influential researchers whose work ethic is surpassed by few others. I want to thank you for willing to take a chance on me when I first interviewed for a Masters position in your lab. Since then, I have never stopped learning from you and you have provided me with tremendous wisdom that has allowed me to grow at both a professional and personal level. Regardless of the path I choose to follow, many of the things you have taught me will not be forgotten and will permeate the rest of my life. I suppose I also need to give you a customary thanks for introducing me to my future wife-to-be, it is much appreciated.

I would also like to thank Dr. Robert Gros, who has been instrumental in the development of the research presented in this thesis. Without you, many of the technical and experimental aspects of this work would not exist. I very much appreciate the vast amount of time you spent teaching me intricate technical skills as well as demonstrating the skills necessary to succeed in science. I would also like to thank Dr. Qingping Feng for serving on my advisory committee and providing valuable insight and extremely helpful suggestions throughout my PhD tenure.

Dr. Vania Prado is another mentor who has played a critical role in my professional and personal development over the past few years. I have had the pleasure of witnessing your abilities in scientific research and teaching during my time in the lab. I have been able to learn a lot from you over the years and it has helped me develop into the person I am today. Not only are you a tremendous scientist but you also take a keen interest in the personal lives of students and treat them as if they were your children. You are extremely

approachable and are always willing to assist us with our work. In addition, you are available without fail should we need help regarding any matter, whether it is lab-related or personal. The connection that you form with students makes you a very special mentor and I am certain that I will maintain a close relationship with you over the years to come.

In addition, I would like to thank the research technicians in the Prado lab, Jue and Sanda, for helping with all the extremely important everyday things that ensure smooth sailing in the lab. Furthermore, the countless students and trainees who have come and gone through the Prado lab deserve more than a simple thanks. Without these friends and colleagues, not only would this work have suffered, but lab life would have been quite boring. Many thanks to Ivana, AraC, Fabi, Patate, Raul, Andre, Eneas, Aline, Val, Benny and Fofão, to name a few. A special thanks to my "sister" Dora who was always there to listen when needed and Flavio, who really helped hone my appreciation for wine and amazing Brazilian food (including feijoada).

Additionally, I need to thank all the childish students in the lab, including Momo and Bilau, who helped confirm that I am the mature one. All of you have provided me with very important pieces of wisdom. Firstly, Momo, you have shown me that it is extremely important to always have a research question in mind before you do an experiment. In addition, I am glad you were around to teach me "Momo's pronunciation" for those English words. But in all honesty, I was very impressed with how quickly you were able to establish yourself not only in the lab, but also in Canadian society, especially coming from Kuwait (where you don't even have sushi!). You have been a great friend since I have known you and given me some amazing life advice. All the sushi, caviar, wings and Bolognese we have enjoyed in order to maintain our physique will not be forgotten. I was hoping to give you a true Canadian experience and hunt some moose with you, but I suppose that ship has sailed. Bilau, you have shown me that it is not how hard you work, but rather how well you can delegate to the minions, that makes you successful. You have also shown me that it is never a good idea to go to the gym wearing "Parfum de biryani."

In order to be successful in life, certain qualities are absolutely critical. These include integrity, discipline, curiosity, work ethic, and organization. I believe that these qualities are instilled in us when we are children and I would like to thank my parents, Aqiq and Sheeba Roy, for imparting these qualities to me. Without your hard work and sacrifice, I would not have gotten this far in life and I owe my deepest gratitude to both of you. I would also like to thank my sisters, Minaqshi and Arsha, as well as my brother, Finhaas, for always supporting me and being there for me when I needed them.

Last, but definitely not least, I need to convey my deepest appreciation and thanks to my fiancée, Ciric. You are always willing to put me first and do everything in your power to make my life easier. I appreciate the constant encouragement you provided with just a hint of doubt, it really turned out to be the perfect recipe. The fact that I can always seek solutions to my scientific and personal problems over fine food and wine with you has been instrumental to my success and I look forward to more of the same for the rest of our lives. Thank you for your endless support and understanding, not to mention willingness to let me overindulge in food/drink/shisha to help me "relax" and "focus." I am truly thankful for having you in my life and love you immensely because you have the uncanny ability to make everything better, even from far, far away. I dedicate this thesis to you.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Full name
α7nAChR	α7 nicotinic acetylcholine receptor
ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitor
ACh	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotropic hormone
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
ANS	Autonomic nervous system
Ara-C	Cytosine-D-arabinofuranoside
ARBs	Angiotensin receptor blockers
AT	Atropine

ATP	Adenosine triphosphate
β-MHC	β-myosin heavy chain
B.W.	Body weight
BAC	Bacterial artificial chromosome
BChE	Buturylcholinesterase
Ca^{2+}	Calcium
CAD	Coronary artery disease
CHF	Chronic heart failure
CaMKII	Calcium/calmodulin-dependent protein kinase
Carb.	Carbachol
cChAT	Cardiomyocyte-specific ChAT KO mouse line
ChAT	Choline acetyltransferase
ChR2	Channelrhodopsin 2
CHT1	High-affinity choline transporter

CICR	Calcium-induced calcium-release
CK	Creatine kinase
Cr	Creatine
cVAChT	Cardiomyocyte-specific VAChT KO mouse line
ECG	Electrocardiography
EDV	End diastolic volume
EF	Ejection fraction
ET-1	Endothelin 1
EYFP	Enanced yellow fluorescent protein
F-actin	Filamentous actin
Floxed	Flanked by loxP
FS	Fractional shortening
GFR	Glomerular filtration rate
GPCR	G protein coupled receptor

GRK2	G protein coupled receptor kinase 2
GRK5	G protein coupled receptor kinase 5
H.W.	Heart weight
H&E	Hematoxylin & eosin
HC-3	Hemicholinium-3
HF	Heart failure
HPLC-ED	High performance liquid chromatography with electrochemical detection
IL-1β	Interleukin 1β
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
ISO	Isoproterenol
KO	Knockout
LV	Left ventricle
LVIDd	Left ventricular internal end-diastolic dimension

LVIDs	Left ventricular internal end-systolic dimension
M_2-AChR	Type 2 muscarinic acetylcholine receptor
MCU	Mitochondrial calcium uniporter
MMP	Matrix metalloproteinase
mPTP	Mitochondrial permeability transition pore
NCX	Sodium calcium exchanger
NE	Norepinephrine
NEO	Neostigmine
NFAT	Nuclear factor of activated T cells
NICM	Non-ischemic dilated cardiomyopathy
NNCS	Non-neuronal cholinergic system
NO	Nitric oxide
NOS	Nitric oxide synthase
NTS	Nucleus of the solitary tract

NYHA	New York Heart Association
PCr	Phosphocreatine
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHE	Phenylephrine
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMCA	Plasma membrane calcium ATPase
PSNS	Parasympathetic nervous system
PVN	Paraventricular nucleus
PYR	Pyridostigmine
RAAS	Renin-angiotensin-aldosterone system

RGS	Regulators of G protein signaling
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SERCA2a	Sarco-endoplasmic reticulum calcium ATPase
siRNA	Small interfering RNA
SNS	Sympathetic nervous system
SR	Sarcoplasmic reticulum
T.L.	Tibia length
TAC	Transverse aortic constriction
TCA	Tricarboxylic acid
TNFα	Tumour necrosis factor α
TnI	Troponin I
VAChT	Vesicular acetylcholine transporter
VES	Vesamicol

WT	Wild type

Chapter 1

1 Introduction

Acetylcholine (ACh), a phylogenetically ancient signaling molecule, was first identified by Otto Loewi in 1921 as the molecule secreted from the Vagus nerve which acts as the main mediator of chemical synaptic transmission in the parasympathetic nervous system. Since its discovery, it has been implicated in the regulation of many physiological functions in both the central and peripheral nervous systems. The parasympathetic nervous system (PSNS) regulates the heart via the Vagus nerves, which mainly innervate the atria, with some sparse ventricular innervation, [1] and play a crucial role in regulating several aspects of cardiac physiology.

Cholinergic signaling leads to a reduction in heart rate, the contractile forces of the atria and the conduction velocity of both the sinoatrial and atrioventricular nodes. Past evidence suggests that, under physiological conditions, parasympathetic tone is the main regulator of heart rate and cardiac activity [2, 3]. Therefore, it is likely that, under pathological conditions, cholinergic tone is required to balance sympathetic signaling and prevent a number of known adverse effects associated with increased sympathetic signaling. This may serve to prevent cardiomyocyte remodeling associated with over activation of the sympathetic system, as observed during the activation of neurohumoral responses in heart failure. However, it is unknown whether modulation of cholinergic activity can serve as a new target for the treatment of cardiac disease by delaying the progression of cardiac dysfunction associated with heart failure.

1.1 The heart failure syndrome

Heart failure (HF) is not a disease; rather, it can be characterized as a progressive syndrome and the final outcome of a number of different acute and chronic conditions. It is characterized by significant damage and weakening of the cardiac muscle leading to decreased cardiac output. The prevalence of HF is very high with more that 5.1 million people over the age of 20 suffering from HF in the US alone [4]. Despite significant advancements in clinical care of HF patients, this syndrome continues to be an important contributor to high morbidity (greater than 1 million hospital discharges), mortality (greater than 50,000 deaths) and economic burden ($30.7 billion) in the US, according to the latest update on heart disease and stroke [4].

Although heart failure leads to significant changes in the cardiac muscle, robust defects are also observed in other organs that can be affected by many of the physiological changes associated with the progression of the HF syndrome. This includes an increase in fluid retention in the lungs, which worsens as the left ventricular function declines and there is a greater increase in pulmonary venous pressure [5, 6]. This accumulation of fluid in the lungs leads to impairment of gas exchange and contributes to the clinical symptoms of heart failure, which include shortness of breath, especially during strenuous work [7, 8] and even correlates with prognosis in heart failure patients [9]. In addition, the decrease in cardiac output during heart failure has a pronounced effect on the kidneys, which begin to retain salt and water [10]. This further exacerbates the pathophysiological response as it leads to an increase in preload and thereby places further strain on the failing cardiac muscle.

Clinically, heart failure is associated with a progressive decline in cardiac function, which leads to increased morbidity and mortality. Furthermore, it is important to note that the significant molecular and structural remodeling processes, which are initially activated in order to maintain homeostatic cardiac function, lead to a further aggravation of cardiac remodeling.

1.2 Regulation of cardiac hemodynamics

In a healthy heart, there is a fine balance between the mechanisms regulating the filling and ejection phases of cardiac contractility. Cardiac performance is mainly determined by five key mechanisms which are active in the cardiovascular system; heart rate, inotropy, lusitropy, preload and afterload. These mechanisms are intricately connected and, as such, defects in any of these will lead to a rapid and progressive decline in the function of the entire heart.

The heart rate is determined by both sympathetic and parasympathetic signaling at the pacemaker region of the SA node where the wave of depolarization leading to contraction is initiated [11]. This wave is then propagated through the atria, the AV node and finally the Purkinje system to induce contraction of atrial and ventricular tissue, in succession, to produce systole [12].

Inotropy and lusitropy, which refer to the contraction and relaxation of ventricular tissue, respectively, are mechanisms modulated by the biochemical and biophysical properties of ventricular cardiomyocytes. As such, they are drastically sensitive to the molecular changes that occur in cardiomyocytes during heart disease. These mechanisms can be considerably influenced by several different variables including changes in calcium re-uptake into the sarcoplasmic reticulum (SR) [13], as well as changes in the calcium sensitivity of contractile proteins, including troponin [14, 15]. In addition, changes in cytoskeletal proteins [16] and ventricular geometry (e.g. thickness of the ventricular walls) can also have a significant effect on the contractile ability of the myocardium [17]. Although heart rate, inotropy and lusitropy are all regulated by the cardiac muscle, both preload and afterload are modulated by changes in venous blood flow. Preload is a measure of the amount of blood returning to the heart from venous circulation. Afterload refers to the amount of blood remaining in the heart following the end of systole and can change depending on arterial pressure, especially in the aorta. Under physiological conditions, there is a fine interplay between venous return of blood to the heart and

cardiac output. Stroke volume is increased when more blood returns to the heart and can be decreased when the amount of venous return is low enough to reduce atrial pressure [18, 19].

Many of these mechanisms, which regulate cardiac output in a healthy heart, appear to be altered in heart failure and lead to the observed hemodynamic abnormalities. The initial insult can play a large part in determining the specific hemodynamic changes that will occur and, therefore, characterize the type of heart failure observed in patients. There are several distinct manifestations of the heart failure syndrome and these are classified according to whether the initial abnormality affects the hemodynamic parameters during systole or diastole.

1.2.1 *Systolic and diastolic heart failure*

Systolic heart failure is caused by several different initial insults, including myocardial infarctions, can lead to damage of the ventricular walls [20]. Ventricular wall damage is associated with enlargement of the ventricles and molecular remodeling of the cardiac myocytes, which leads to cardiomyocyte elongation and reduced myocyte shortening capacity [21, 22]. Conditions that damage or weaken the myocardium can serve as the initial trigger for cardiomyocyte remodeling and the molecular changes associated with altered myocyte function lead to an overall decrease in ventricular ejection fraction and systolic heart failure [23, 24]. These include insults that have a regional effect (e.g. myocardial infarction), or an effect on the entire cardiac muscle (e.g. viral and toxic myocarditis) [20, 25, 26]. All of these insults lead to an increase in ventricular cavity volume due to an elongation of cardiomyocytes (i.e. eccentric hypertrophy) [27]. The changes in the architectural and anatomical structure of the heart in systolic heart failure lead to inefficient contraction of the ventricular muscle and result in impaired ventricular ejection.

Conversely, diastolic heart failure is caused by reduced ventricular cavity size, which often results from thickening of the ventricular cardiomyocytes [28]. Although ventricular cavity volume is significantly increased in systolic heart failure, it often

appears to be unaltered, or minimally changed, in patients with diastolic heart failure [29, 30]. This is partly due to the fact that hypertrophic cardiomyopathies, such as hypertension and diabetes, are the leading cause of diastolic HF [29]. The cardiomyocytes undergo concentric, as opposed to eccentric, hypertrophy in diastolic HF and a thickening, rather than elongation, of cardiac myocytes is observed [27]. In diastolic HF, there is decreased ventricular compliance during diastole due to thickening of the ventricular walls and this increase in rigidity of the ventricular walls results in abnormal relaxation and passive stiffness [31]. Diastolic heart failure leads to a subsequent increase in venous pressure, which ultimately affects pulmonary pressure and promotes the development of pulmonary edema, a clinical symptom often observed in patients with diastolic HF [32].

1.3 Hemodynamic changes in heart failure

It is important to recognize that several mechanisms contribute to impaired cardiac function in heart failure. Systolic and diastolic dysfunction cannot simply be linked to changes in inotropy or lusitropy, respectively. Rather, the activation of neurohumoral mechanisms meant to maintain cardiac output leads to altered ventricular hemodynamics through a variety of pathways and contributes to a very complex heart failure phenotype. Depending on whether the initial insult leads to systolic or diastolic HF, the myocardium responds by undergoing either eccentric or concentric hypertrophy, respectively. Eccentric hypertrophy leads to impairment in ventricular filling and a decrease in EDV due to the fact that left ventricular dilation increases wall stress during diastole [33, 34]. Furthermore, it leads to inefficient ejection of blood during systole as the altered structure of the myocardium and increased ventricular diameter contribute to further mechanical disadvantage. The extent of contractile dysfunction associated with eccentric hypertrophy can also be affected by the presence of fibrosis following myocardial damage, which leads to cardiomyocyte necrosis and decreases myocardial compliance during all phases of the cardiac cycle [35, 36].

This enhanced fibrotic response also plays a significant role in heart failure associated with concentric hypertrophy [37], in which the thickening of the cardiomyocytes, and thus the ventricles, leads to impairment in both the filling and ejection capacity of the heart [38]. This is due to the decrease in ventricular volume associated with concentric hypertrophy, which has the effect of reducing stroke volume and increasing end diastolic pressure. Over time, the reduced ventricular compliance associated with concentric hypertrophy and diastolic dysfunction results in systolic dysfunction and the onset of heart failure. Regardless of the initial dysfunction, cardiac remodeling and hypertrophy often progress to ventricular dilation and a severe decline in cardiac output (Fig. 1.1).

Following initial cardiac remodeling and reduction in ventricular contractility and ejection, increased residual blood volume (i.e. end-systolic volume) leads to an increase in end-diastolic volume, which activates hemodynamic responses involved in altering both preload and afterload to maintain homeostatic cardiac output. In the event of impaired ventricular filling, as is observed in diastolic dysfunction, vasoconstriction and fluid retention in the kidneys are activated in order to increase preload and afterload, respectively [39].

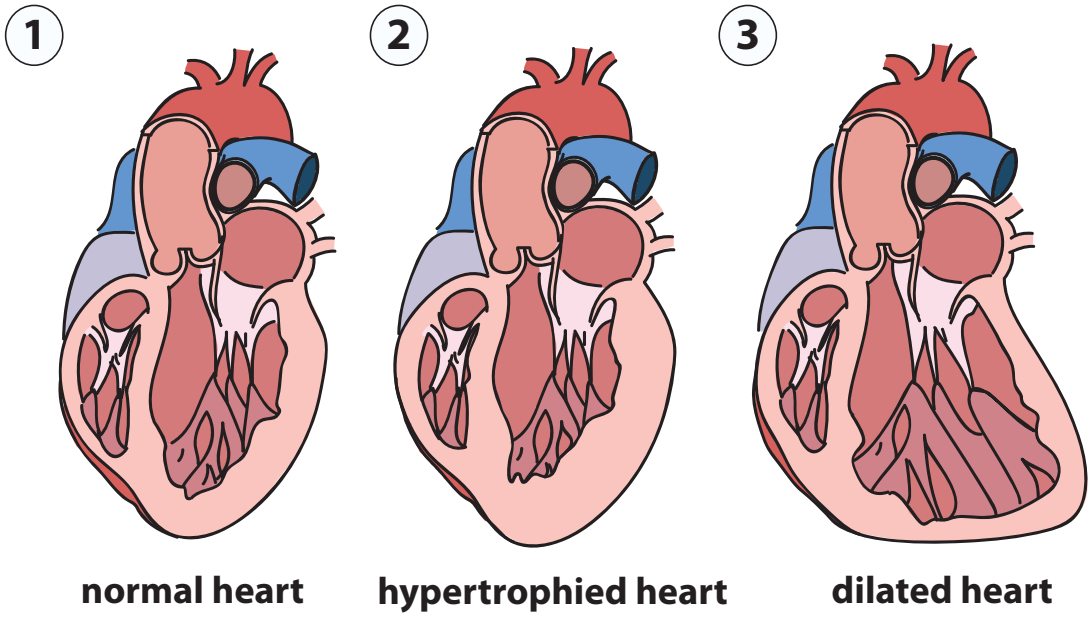
The hemodynamic abnormalities observed in HF patients can be altered by neurohumoral responses including vasoconstriction, fluid retention and changes in proliferative signaling. However, these responses tend to have maladaptive long-term consequences, which lead to a progressive decline in cardiac function and greater myocardial remodeling in heart failure.

1.4 Neurohumoral responses to decreased cardiac output

The initial changes in ventricular hemodynamics, including reduced arterial filling and increased accumulation of blood in venous circulation, activate the neurohumoral response in order to maintain cardiac homeostasis. Although these responses are critical

Figure 1.1 - Progression of cardiac remodeling in heart failure. (1) A healthy heart can regulate short-term changes in cardiac output and has the ability to maintain the metabolic demands of the body. **(2)** The presence of cardiovascular stressors or disease leads to activation of neurohumoral responses to maintain ventricular hemodynamics, which results in pathological hypertrophy associated with the activation of various molecular remodeling processes. **(3)** Prolonged hypertrophy and activation of neurohumoral responses, including enhanced sympathetic tone, leads to ventricular dilation characterized by an enlargement of the ventricular cavity. This leads to a further decline in cardiac output and heart failure.

Figure 1.1 - Progression of cardiac remodeling in heart failure.



in regulating cardiac function during the short-term, they can also exacerbate and enhance the progression of heart failure.

Interestingly, the decrease in arterial filling observed following the initial cardiac insult activates pathways, which lead to an increase in cardiac output, vasoconstriction of blood vessels and fluid retention in the kidneys through inhibition of sodium and water excretion [40]. These responses are commonly activated when the cardiovascular system is challenged over the short-term through either exercise or increased blood loss, as seen during hemorrhaging [41, 42]. However, in heart failure, as cardiac function progressively declines, this neurohumoral response persists and becomes maladaptive.

In addition to these changes to the cardiovascular system, long-term changes, including transcriptional changes that can alter cardiomyocyte function and signaling, also take place concurrently. Furthermore, the activation of pro-inflammatory responses play a significant role in altering the progression of heart failure [43, 44] and can evolve slowly over months and years following the initial induction of cardiac disease.

1.4.1 *Cardiac stimulation to increase cardiac output*

Similar to the neurohumoral response observed in exercise, the increase in cardiac output in heart failure is modulated by activation of autonomic centers in the brainstem. In response to decreased arterial filling, activation of the baroreceptor reflex leads to a subsequent increase in sympathetic activity and reduction in parasympathetic tone [45, 46]. This increase in noradrenergic signaling leads to an increase in ventricular ejection by enhancing inotropic responses, augmenting ventricular filling by affecting lusitropy and also increasing cardiac output by accelerating heart rate through its positive effects on chronotropy [47, 48]. An increase in cardiac contractility decreases end-systolic volume by enhancing ventricular ejection. Importantly, the positive effects on cardiac contractility are not solely due to the effects of norepinephrine on cardiac myocytes; rather, other regulatory mediators including angiotensin II [49], vasopressin [50] and endothelin [51] also play minor roles in increasing the contractile response. Similarly,

enhanced lusitropic responses can also increase cardiac output by increasing end diastolic volume through greater myocardial relaxation during diastole [52].

Though these effects have a positive influence on cardiac output and maintain the metabolic requirements of all tissues, the long-term activation of these responses leads to a chronic cardiac energy deficit due to consistently elevated cardiac energy utilization that accompanies increased chronotropic and inotropic responses. This leads to a significant decrease in myocardial reserve ATP levels and is thought to contribute to diastolic dysfunction [53] and increased susceptibility of the heart to stress [54].

1.4.2 *Vasoconstriction to maintain blood pressure*

In the event of reduced arterial filling, as seen in heart failure, a major neurohumoral response involves activation of arterial vasoconstriction in order to increase peripheral vascular resistance [55, 56]. This increase in vasoconstriction is activated through sympathetic stimulation, which leads to the release of norepinephrine, downstream activation of α_1-adrenergic receptors on smooth muscle cells and increased blood pressure [57]. During heart failure, this mechanism increases afterload in order to maintain blood pressure. However, the reflex response to increase vasoconstriction can lead to an increase in myocardial energy demand. This increase in energy expenditure is due to the fact that the cardiac muscle must sustain greater contractility in order to maintain normal cardiac output against greater resistance. This places further strain on the damaged cardiac muscle and, once again, leads to a decline in cardiac function.

Aside from its negative effects on cardiac muscle, this increase in vasoconstriction also has maladaptive effects in other organs as it leads to a reduction in the amount of blood flow throughout the body. This can contribute to an enhancement of clinical symptoms in patients with end-stage HF, including anemia [58].

1.4.3 *Fluid retention to maintain cardiac output*

In the event of cardiogenic shock, which is observed under conditions of severe left ventricular dysfunction (e.g. following myocardial infarction or myocarditis), there is a significant and drastic decline in blood pressure [59]. In order to counteract this extreme decrease in arterial filling, the kidneys retain salt and water. This restores blood volume and increases preload, which can help maintain cardiac output even under conditions where the myocardium is damaged [39]. However, due to the long-term deficits in cardiac output observed in heart failure, these responses become detrimental because the increase in preload further augments the problems associated with elevated systemic and pulmonary venous pressures. In fact, sodium and water retention by the kidneys serves as a cause for edema, one of the major clinical symptoms observed in heart failure [60]. In severe HF, the significant decrease in cardiac output leads to a subsequent reduction in glomerular filtration rate (GFR) and contributes to increased sodium retention, and hence water retention, in the plasma [61]. Additionally, vasoconstriction of renal efferent arterioles and increased sodium reabsorption by renal tubules also leads to significant fluid retention in heart failure [61].

1.5 Mediators of the neurohumoral response

Many of the signaling cascades activated during the neurohumoral response contribute to maintaining hemodynamic homeostasis; however, these cascades activate pathological hypertrophy and lead to the progression of cardiac dysfunction and heart failure. The first major neurohumoral response activated in heart failure involves a drastic increase in secretion of norepinephrine by sympathetic nerves [62]. However, in addition to these changes in sympathetic activity, there is also a marked activation of several other signaling pathways.

1.5.1 *Renin-angiotensin-aldosterone system (RAAS)*

The RAAS system plays a crucial role in modulating blood pressure and fluid retention and can, therefore, be activated to maintain homeostasis during changes in blood volume, as observed in heart failure [63]. Under conditions where blood volume is decreased, renin gets secreted from juxtaglomerular cells in the kidneys into the plasma [64, 65]. Renin is then able to activate the conversion of the peptide angiotensinogen, which is secreted from the liver, into angiotensin I [66, 67]. The subsequent conversion of angiotensin I into angiotensin II occurs via the proteolytic actions of the angiotensin-converting enzyme (ACE) in both the blood, where it is found in high concentrations on the surface of endothelial cells, as well as several tissues throughout the body [68-70]. Interestingly, the same enzyme which catalyzes the conversion of angiotensin I into angiotensin II also hydrolyzes the peptide, bradykinin [71-73]. This protein serves as a vasodilator due to its actions on endothelial cells in the vasculature where it induces the production of NO, cAMP and prostacyclins [74-76]. As such, ACE can play a significant role in modulating vascular tone because it can increase production of angiotensin II, a vasoconstrictor, and concurrently hydrolyze the vasodilator, bradykinin.

Angiotensin II has emerged as a multifunctional cytokine that exerts its effect through interaction with two G-protein coupled receptor (GPCR) subtypes, AT_1 and AT_2, which regulate opposing responses [77, 78]. The levels of AT_2 receptors are significantly lower than AT_1 receptors in adult tissues. Although the importance of AT_2 receptors in heart failure is not as well understood, angiotensin II appears to signal via this receptor subtype to induce vasodilation and inhibition of cellular growth [79, 80]. AT_1-dependent signaling leads to potent vasoconstriction through direct effects on vascular smooth muscle cells [81, 82]. In addition, it can also have a small effect on inotropy as it can enhance myocardial contractility [83]. Finally, AT_1 receptor signaling can also activate cardiomyocyte hypertrophy, likely through both mechanical stretching as well as direct effects on ventricular myocytes [84-86].

In addition to its effects in the cardiovascular system, angiotensin II also appears to activate AT_1 receptors in the paraventricular nucleus (PVN) of the hypothalamus and

thereby regulate fluid balance and sympathetic drive in an animal model of congestive heart failure [87]. Pharmacological inhibition of this receptor in the PVN reduces neuronal firing and decreases sympathetic drive and volume accumulation [87]. Furthermore, angiotensin II also plays a major role in regulating fluid reabsorption in the kidneys. It is able to stimulate the transcription and activity of Na^+/H^+ exchangers [88, 89] as well as induce hypertrophy of renal tubule cells [90, 91], which leads to an increase in sodium reabsorption. As sodium absorption increases, water is subsequently taken up from the urine by the tubules, which increases blood volume and facilitates the angiotensin II-mediated increase in blood pressure [92].

Importantly, Ang II appears to have a critical function in several different pathways that regulate the progression of pathological remodeling in cardiac disease. It has direct effects via AT_1 and AT_2 receptors, but it can also stimulate the release of other modulators of the cardiovascular system. These include aldosterone, vasopressin, endothelin, nitric oxide, as well as catecholamines, including epinephrine and norepinephrine.

In a healthy individual, aldosterone secretion is regulated by adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which can then stimulate aldosterone synthesis in the adrenal cortex [93]. In severe heart failure, the high concentration of circulating angiotensin II serves as the major stimulator of aldosterone secretion, especially in the left ventricle [94, 95]. Once produced, aldosterone is secreted into the blood and can act on distal tubules to increase sodium and water reabsorption and increase blood volume as well as blood pressure through this mechanism [93].

1.5.2 *Vasopressin*

Angiotensin II can also stimulate the release of the peptide hormone vasopressin from the posterior pituitary gland, which is normally secreted in response to low blood volume [96]. The major function of vasopressin is to increase water permeability of the renal collecting ducts through V_2 receptor-mediated regulation of aquaporin expression and

distribution, thereby inhibiting water secretion and increasing blood volume [97, 98]. In addition, it can also augment vasoconstriction by enhancing the effects of catecholamines and thereby increasing blood pressure [99]. In heart failure, these potent effects of vasopressin become maladaptive due to the fact that vasopressin secretion is constantly elevated in response to increased levels of circulating Ang II as well as arterial underfilling as a result of decreased cardiac output.

1.5.3 *Nitric oxide (NO)*

Nitric oxide is an important cellular signaling molecule in many different cell types throughout the body, including endothelial cells in the vasculature [100]. It is biosynthesized by three separate nitric oxide synthase (NOS) enzymes using the precursor L-arginine [101, 102]. Interestingly, Ang II also plays a role in inducing pathophysiological responses via NO signaling as exposure to angiotensin II can increase eNOS transcription and NO production [103].

Two of the three isoforms of NOS, nNOS and eNOS, are constitutively active enzymes and play a role in physiological signaling through autocrine/paracrine effects [104]. NO induces its physiological effects, in particular vasodilation, through binding to soluble guanylyl cyclases which leads to the production of cGMP and protein kinase G (PKG), the protein which modulates reuptake of calcium into intracellular stores [105, 106]. Importantly, physiologically relevant concentrations of NO are maintained by eNOS and nNOS, which are activated by calcium and calmodulin [104]. However, the inducible form of NOS (iNOS), which is expressed in endothelial cells, smooth muscle cells as well as macrophages can be activated by immunostimulatory cytokines or infection and produce very high concentrations of NO in a calcium-independent manner [102, 104, 107].

Activation of iNOS in isolated cardiomyocytes by inflammatory mediators is able to inhibit contractility [108]. In fact, experimental heart failure in rabbits has been shown to increase levels of iNOS, which may contribute to contractile dysfunction due to increased

production of superoxides in the heart [109]. iNOS-dependent superoxide production may compound the increased oxidative stress observed due to the stimulatory effects of Ang II on endothelial NADPH oxidase, which leads to the production of hydrogen peroxide and superoxides [110-112]. Importantly, iNOS expression is significantly increased in human heart failure, especially in dilated cardiomyopathy and ischemic heart disease, which suggests a functional role for iNOS signaling in cardiac disease in humans [113].

1.5.4 *Endothelin*

Angiotensin II can mediate secretion of the protein, endothelin, from endothelial cells in the vasculature [114-116]. Endothelin-1 (ET-1) is the major isoform of endothelin in the cardiovascular system and its release can be modulated through the actions of angiotensin II, epinephrine [117], mechanical pressure [118, 119], and high shear stress along the vessel walls [120]. Endothelins are very potent vasoconstrictors and mediate these effects via the ET_A GPCRs in a paracrine manner [121]. ET_A receptors couple to G_q proteins in vascular smooth muscle cells and binding of ET-1 to these receptors induces vasoconstriction through activation of PLC to produce IP_3 and DAG [122]. In addition to its effects on vascular tone, ET-1 also increases cardiac contractility over the short-term [117] while the long-term effects include induction of hypertrophic remodeling in cardiomyocytes [123]. Furthermore, ET-1 has been shown to induce pro-inflammatory responses in different tissues, including the myocardium [124, 125].

Under normal physiological conditions, vascular tone is maintained through a delicate balance between vasodilators and vasoconstrictors, including ET-1. However, the drastic changes observed in cardiac output during heart failure lead to significant activation of several neurohumoral mechanisms. Importantly, it appears that the overactivation of the sympathetic system that occurs early during the onset of heart failure plays a crucial role in the activation of many of these compensatory mechanisms.

1.5.5 *Sympathetic hyperactivity*

The autonomic nervous system is the main regulator of cardiac output. It is accepted that chronic autonomic sympathetic/parasympathetic imbalance plays a crucial role in the development of HF and is a critical modulator of the neurohumoral response [126-129]. Importantly, increased sympathetic tone, coupled with decreased parasympathetic drive, is seen even in the early stages of CHF [130, 131].

Several lines of research implicate the over-activation of the sympathetic nervous system in patients as a major contributor to cardiac remodeling [62, 132]. This is associated with poorer prognosis and higher morbidity and mortality [133], as increased sympathetic tone may help to preserve cardiac function initially, but then may contribute to remodeling [128, 134]. The activation of neurohumoral mechanisms, including increased SNS signaling, is a hallmark of heart failure resulting from homeostatic regulation following the initial decrease in cardiac output [128, 134]. SNS hyperactivity is observed in several cardiac diseases including hypertension, where patients display a significant increase in SNS activity, a phenomenon which appears to contribute to altered blood pressure regulation as well as left ventricular dysfunction [135]. Importantly, decreasing SNS signaling through activation of the baroreflex has been shown to increase survival in an animal model of chronic HF [136], thus suggesting that enhanced sympathetic signaling plays a critical role in the progression of cardiac dysfunction.

Norepinephrine, secreted by the sympathetic nervous system, binds to adrenergic receptors in the heart as well as the vasculature and kidneys. As such, it plays a significant role in modulating maladaptive responses in heart failure. In the heart, norepinephrine can bind to β_1-adrenergic receptors, the major subtype in cardiac tissue, and activate second messenger signaling via the stimulatory G-protein, G_s, which activates adenylyl cyclase and cAMP production [137]. The noradrenergic-mediated inotropic, lusitropic and chronotropic responses observed following activation of the β_1 adrenergic receptor serve to maintain cardiac output [138], but also mediate pathological cardiac hypertrophic responses and induce myocyte apoptosis, thus leading to further weakening of the failing cardiac muscle [137, 139].

In the vasculature, norepinephrine binds to α-adrenergic receptors, which are coupled to G_q proteins. As such, activation of α_1-adrenergic receptors activates production of IP_3 and DAG, which induce vasoconstriction in vascular smooth muscle cells through IP_3-mediated calcium release from intracellular stores and activates DAG-mediated signaling via protein kinase C (PKC) [140]. The arterial vasoconstriction mediated by noradrenergic signaling can increase blood pressure and promote both sodium and fluid retention in the kidneys.

Modulation of the autonomic nervous system has become an important target in decreasing morbidity and mortality in heart failure patients. Currently, β-adrenergic receptor blockers, in combination with angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs), are used as a first-line treatment for HF [141]. β-blockers work by inhibiting the effect of norepinephrine, released from sympathetic neurons, on β-adrenergic receptors in both the heart and vasculature and can thereby decrease heart rate and lower blood pressure through vasodilation. This reduces the pressure placed on the heart and thus preserves cardiac function. The chronic use of β-blockers has been quite successful and has been shown to increase cardiac function and reduce left ventricular remodeling and mortality in patients, with third-generation non-selective β-blockers decreasing mortality more than second-generation β_1-selective drugs (34% vs. 40%, respectively) [142-144].

The importance of modulating sympathetic tone in left ventricular hypertrophy and cardiomyopathy is further highlighted by the fact that autoantibodies against the β_1-adrenergic receptor are found in patients with both cardiac hypertrophy and dilated cardiomyopathy [145, 146]. The cause and effect relationship between the production of autoantibodies and cardiomyopathy has not yet been fully elucidated. However, the presence of these autoantibodies has been shown to activate signaling cascades downstream of β_1-adrenergic signaling in cultured myocytes and may contribute to the cardiac dysfunction observed in cardiomyopathy [147]. It is also possible that, in addition to activation of signaling pathways downstream of adrenergic receptor activation, the presence of these autoantibodies also leads to autoimmune-mediated cardiac damage.

In addition to the positive effects observed following inhibition of direct actions of sympathetic signaling in the heart, positive outcomes have been observed following renal sympathetic denervation in patients with heart failure. Activation of the renal sympathetic nerves in heart failure leads to increased peripheral vascular resistance and ventricular remodeling, partly due to higher levels of angiotensin II. Renal denervation has shown promise in human heart failure patients, as there was a trend towards improvement in exercise capacity and LV hypertrophy [148, 149]. Furthermore, independent of its blood pressure lowering effects, renal denervation has been shown to have a positive effect on atrial fibrillation [150] and heart rate reduction [151], both of which have been shown to play a critical role in reducing morbidity and mortality in HF patients.

1.6 Cellular and molecular abnormalities in response to neurohumoral signaling

The activation of all the pathways that regulate neurohumoral signaling as well as the chronic increase in cardiac volume, which occurs in order to compensate for hemodynamic changes, leads to significant cellular and molecular abnormalities in failing hearts. These cellular changes constitute the significant cardiomyocyte remodeling observed in heart failure and lead to further defects in ventricular ejection during systole and/or impaired filling of the ventricles during diastole. The abnormalities that contribute to these defects can be characterized as functional changes wherein normal cardiac proteins and structures responsible for proper functioning of the cardiac muscle are altered. In addition, the changes at the cellular level also lead to changes in the architectural structure of the heart, likely as the result of chronic exposure to neurohumoral signaling at the level of the cardiac muscle.

1.6.1 *Cardiac contraction and relaxation*

The heart is composed of several different cell types, including endothelial cells, fibroblasts as well as vascular smooth muscle cells and cardiomyocytes. There are several

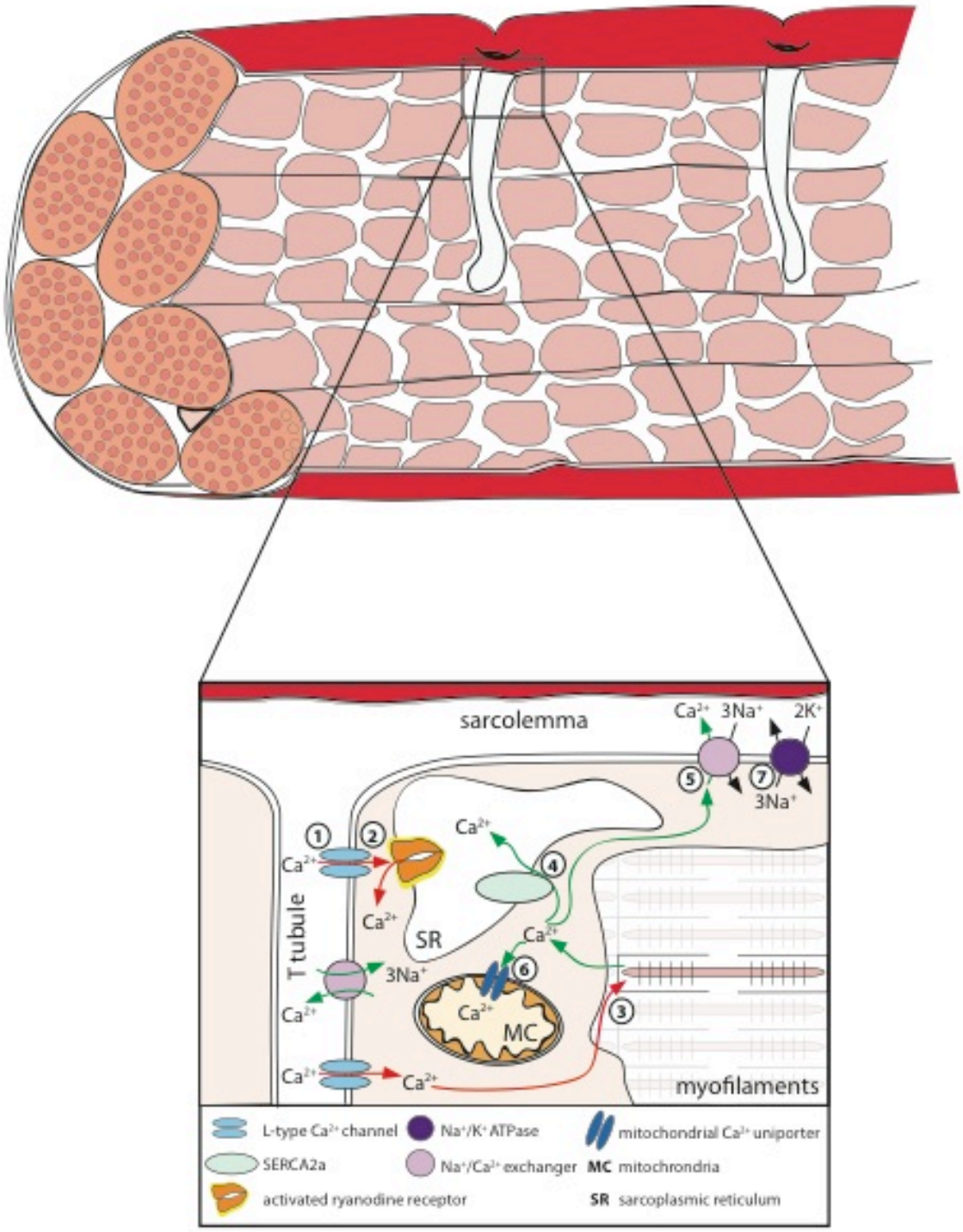
types of cardiomyocytes present in the heart, including specialized myocytes capable of conducting action potentials that regulate chronotropy via intracellular cAMP signaling [152]. In contrast, inotropy and lusitropy are dependent on the function of working cardiomyocytes, which are organized in a branched network. Synchronous contraction is maintained via specialized cell-cell junctions that allow action potentials to travel between adjacent cardiomyocytes without interruption [153]. Due to the fact that cardiac myocytes are highly active, contracting cells, nearly half the volume of these cells is composed of contractile proteins organized in myofibrils. Much of the remaining cellular volume is occupied by mitochondria, which provide the energy required for the maintenance of cardiac work.

The myofibrils are composed of myofilaments, which contract in ventricular myocytes following direct activation by the second messenger calcium (Ca^{2+}) during systole [154] (Fig. 1.2). Importantly, in order to maintain synchronous contraction of the entire cardiomyocyte, tubular invaginations known as transverse tubules (T-tubules) are present throughout the cell and allow for proper excitation-contraction coupling by regulating calcium influx via the L-type Ca^{2+} channels [155-157]. Importantly, in heart failure, where there is a decrease in systolic Ca^{2+} transients, there appears to be an upregulation of L-type Ca^{2+} channel density as well as probability of opening, suggesting a response to maintain intracellular Ca^{2+} currents ($I_{Ca,L}$) and preserve cardiac contractility [158, 159]. T-tubules also allow exposure to the extracellular environment and, in combination with the plasma membrane, can mediate excitation-contraction coupling and regulate cell signaling mediated by ligand binding to receptors on the extracellular face of the membranes [160-163].

In addition to the T-tubules and plasma membrane, there are also subcellular structures within the cardiomyocytes that play key roles in regulating excitation-contraction coupling in these cells. One of these structures is the sarcoplasmic reticulum (SR), which

Figure 1.2 - Mechanisms regulating calcium-induced calcium release and excitation-contraction coupling. (1) Action potentials and initiated at the SA node and travel along the sarcolemma and activates voltage-gated L-type Ca^{2+} channels in T-tubules. **(2)** Ca^{2+} influx into the cytosol activates ryanodine receptors (RyRs) on the sarcoplasmic reticulum (SR) and induces Ca^{2+} release from the SR. **(3)** Cytosolic Ca^{2+} binds to Troponin C and induces contraction of myofilaments. **(4)** Following systolic contraction, intracellular Ca^{2+} is transported back into the SR by the sarco/endoplasmic reticulum ATPase (SERCA2a) where it is sequestered until the next contraction. Cytosolic Ca^{2+} is also extruded from the cell via the Na^{+}/ Ca^{2+} exchanger **(5)** and a small amount is taken up by the mitochondria via the mitochondrial Ca^{2+} uniporter (MCU) **(6)**. **(7)** The Na^{+}/K^{+} ATPase transports Na^{+} out of the cell in order to maintain resting membrane potential.

Figure 1.2 - Mechanisms regulating calcium-induced calcium release and excitation-contraction coupling.



is the main intracellular store for Ca^{2+} in myocytes and is responsible for the majority of cytosolic Ca^{2+} that induces contraction [164-166]. All of these structures work together to deliver calcium to the contractile proteins of the sarcomeres during systole.

1.6.2 *Calcium transport in cardiomyocytes*

Calcium entry into the cardiomyocyte upon initiation of contraction is dependent on the activation of L-type calcium channels in the T-tubules and plasma membrane, which allow for the passive diffusion of calcium into the cytosol down its electrochemical gradient [155-157]. Importantly, although extracellular calcium is the major source for contractility in embryonic hearts, where the contractions are weak and develop slowly [167, 168], the extracellular calcium in an adult cardiomyocyte is only sufficient to activate contraction in a small population of sarcomeres. As such, calcium release from intracellular stores, a process dependent on calcium influx from the extracellular environment, is necessary for the development of proper tension and contractility.

Calcium-induced calcium release (CICR) involves release of Ca^{2+} from SR stores into the cytosol through cardiac ryanodine receptors (RyR) on the SR membrane, downstream of Ca^{2+} influx via the L-type Ca^{2+} channels on the T-tubules [166]. Notably, calcium release channels in the cardiomyocytes are highly regulated and phosphorylation by calcium/calmodulin-dependent protein kinase II (CaMKII) can inhibit channel opening and play a protective role under conditions of calcium overload [169]. RyR on the SR can be phosphorylated by PKA following sympathetic activation, an event observed during heart failure, which results in increased calcium release from the SR and a positive inotropic response [170-172]. This leads to myocyte damage due to calcium overload in cardiomyocytes and may contribute to the adverse effects on cardiac function observed in HF following long-term activation of neurohumoral signaling [171].

In addition to RyR, IP_3-gated receptors are also present on the SR membrane [173, 174] and can trigger Ca^{2+} release from intracellular stores [175, 176]. They are thought to

regulate development of arrhythmias by increasing diastolic Ca^{2+} levels as well as pathological signaling downstream of neurohumoral activation [177-180].

1.6.3 *Regulation of cardiomyocyte contractility*

Cardiomyocyte contraction occurs through movement of two sets of filaments, which slide against each other and shorten during systolic contraction. This theory, known as the sliding filament theory, utilizes calcium secreted from intracellular SR stores to induce contraction in cardiomyocytes [181]. The thin and thick myofilaments that compose the sarcomeres partially overlap under resting conditions and slide by one another during contraction to generate tension and shorten the muscle [182]. Sarcomere length is regulated by the amount of overlap between the thin and thick filaments and appears to play a critical role in determining the intensity of excitation-contraction coupling and, hence, the efficiency of contraction [183]. The contractile process is dependent on the interaction between myosin on the thick filaments and actin on the thin filaments, which form a cross-bridge during sarcomeric contraction [182]. The cross-bridge formation that regulates contractility of the sarcomeres requires the presence of cytosolic calcium, with the SR serving as the major source of intracellular calcium for this process [184, 185]. Once released into the cytosol, calcium binds to the troponin complex on the thin filament, which consists of three proteins including troponin I (inhibitory subunit), troponin T (tropomyosin-binding subunit), and troponin C (calcium-binding subunit) [186-189]. This complex regulates the interaction between the thick and thin filaments of filamentous actin (F-actin) which comprise the myofilaments in the sarcomeres [186]. Upon binding to calcium, troponin C causes a rearrangement of regulatory proteins and binds to the inhibitory troponin subunit (TnI) more strongly thus causing it to dissociate from actin [190, 191]. This allows cross-bridge formation between actin and myosin, the motor protein responsible for contraction of the sarcomeres and cardiomyocyte shortening [192, 193]. Importantly, the amount of calcium entering the cytosol during systole under basal conditions is only sufficient to bind approximately half the troponin C molecules present in the cardiomyocyte. This allows the intensity of the contractile response to be modulated by interventions that alter the amount of calcium released from the SR during CICR. Furthermore, it also implies that the activity of both

tropomyosin and the troponin complex play an important role in modulating tension development during contraction.

During systole, the energy for contraction of the sarcomeres, and hence the cardiomyocytes, is provided by ATP hydrolysis at the catalytic site of the myosin head, a process mediated by an actin-activated ATPase found on the myosin head [194]. In order for the sarcomeres to return to the relaxed state during diastole, interdigitation of actin and myosin must be inhibited, a process dependent on ATP binding to the catalytic site of myosin [195]. This sarcomeric relaxation is dependent on the export of Ca^{2+} from the cytosol in order for troponin C to return to its resting state and inhibit actin-myosin interaction. In order for the cell to maintain steady state calcium levels following systole, cytosolic calcium must be effluxed across the plasma membrane or into the SR, a process that occurs against the concentration gradient for this ion.

In order to pump calcium into the extracellular environment, the myocyte utilizes both the plasma membrane calcium pump ATPase (PMCA) as well as the sodium/calcium (Na^{+}/Ca^{2+}) exchanger (NCX), which is responsible for the majority of calcium efflux due to its greater capacity for calcium than PMCA [196, 197]. The actions of PMCA are dependent on ATP hydrolysis which provides the energy to power calcium transport across the plasma membrane [198, 199]. Although PMCA does not play a major role in regulating calcium efflux during diastole, it may play a role in cardiac remodeling as it has been shown to regulate cardiomyocyte hypertrophy following hyperadrenergic stimulation, *in vitro* [200].

The NCX is an antiporter, which utilizes the sodium gradient to translocate three sodium ions into the cell in order to expel one calcium ion across the plasma membrane against its electrochemical gradient [201, 202]. The NCX maintains normal steady state intracellular calcium levels by effluxing a similar concentration of calcium as that which enters the cell via the L-type calcium channels. However, in NCX knockout mice, normal excitation-contraction coupling is maintained through a compensatory decrease in Ca^{2+} influx [203]. Importantly, NCX is activated via phosphorylation by PKA and PKC, a

characteristic that is critical in preventing calcium overload in the cell [204, 205]. Interestingly, in the event of calcium overload, which often occurs in failing hearts, the increase in calcium efflux through the NCX can lead to production of after-depolarizations and ventricular fibrillation due to the increased influx of sodium, which has electrogenic properties [206, 207]. In fact, the most important cause of sudden cardiac death in heart failure is this ionic current generated by increased intracellular sodium concentrations [208, 209].

In addition to calcium efflux into the extracellular space via plasma membrane transporters, the SR also plays a crucial role in removing the majority of cytosolic calcium in order to relax the sarcomeres [210]. The sarcoplasmic reticulum calcium pump (SERCA2a) transports calcium into the SR following systole, a process dependent on ATP hydrolysis. In order to maintain low concentrations of calcium in the SR lumen, calcium is bound to calsequestrin in the SR [211]. The sequestration of calcium by the calsequestrin protein is crucial in maintaining normal calcium stores and thus cardiomyocyte function as altered intracellular calcium stores leads to pathological responses [212, 213]. Importantly, movement of calcium across the SR membrane does not generate an electrical current due to the presence of anion channels on the SR membrane that allow anion transport along with calcium.

It is important to note that the energetic imbalance observed in the failing heart, where there is a deficiency in reserve ATP levels, leads to defective function of the SERCA2a pump. Furthermore, there is a decrease in the expression of SERCA2a in hypertrophied cardiomyocytes, which can inhibit calcium uptake into the SR and thereby impair relaxation during diastole, a process that further exacerbates cardiac dysfunction [214, 215]. The decrease in calcium movement into the SR via SERCA2a is of even greater importance in heart failure because there is significant activation of other processes that contribute to calcium overload and development of both atrial and ventricular arrhythmias in the failing myocardium.

In an attempt to reduce cytosolic calcium levels, mitochondria in failing cardiomyocytes begin to accumulate excess calcium and have been shown to sequester very large amounts of calcium [216, 217]. Calcium enters the mitochondria through the mitochondrial Ca^{2+} uniporter (MCU) and activates enzymes that play key roles in the tricarboxylic acid (TCA) cycle [218-220], which leads to an increase in NADH production, and consequently ATP levels [221]. Normally, calcium uptake and release from mitochondria play a very minor role in excitation-contraction coupling due to the slow turnover of mitochondrial calcium transport [222, 223]. The mitochondria can sequester significant amounts of Ca^{2+}; however, beyond a certain threshold, the mitochondria are unable to regulate [Ca^{2+}] in the matrix and this leads to further mitochondrial overload [224]. This increase in mitochondrial [Ca^{2+}] leads to opening of the mitochondrial permeability transition pore (mPTP) and cell death [225, 226]. mPTP opening further abrogates the energetic deficits because it leads to uncoupling of oxidative phosphorylation [227]. In fact, the negative effect of this process is two-fold as mPTP opening leads to an increase in the production of reactive oxygen species (ROS), which can further perpetuate the molecular changes observed in ischemia/reperfusion injury and heart failure [228-230]. Together, these changes may serve as the molecular basis for the progressive decline in cardiac contractility observed following activation of neurohumoral mechanisms in heart failure.

1.6.4 *Cardiac energetics*

Under normal conditions, the heart maintains a large pool of reserve energy in the form of phosphocreatine (PCr), which can then be used by the creatine kinase (CK) enzyme to generate ATP and drive energy-requiring reactions in the heart [231]. In addition to PCr, glycogen also serves as an energy store in the heart and can generate ATP via glycolytic and oxidative metabolism [232].

Creatine (Cr) is not produced in excitable tissues like the heart but rather, it is transported into cardiomyocytes through the actions of a facilitated creatine transporter against its concentration gradient [233]. Importantly, Cr transporter levels in the failing myocardium

are significantly decreased, and may explain the observed decrease in [Cr][234-236]. The decrease in Cr levels in the failing heart leads to a decrease in cardiac energetic reserves and likely contributes to the decreased [ATP] observed in heart failure because it occurs earlier in cardiac disease than the decrease in ATP levels [237, 238]. In patients with heart failure, the phosphocreatine-to-ATP (PCr/ATP) ratio is significantly reduced and can serve as a predictor of mortality [239, 240]. Furthermore, absolute levels of both ATP and phosphocreatine levels are significantly reduced [241-243]. This decrease in PCr/ATP ratio is especially profound in the subendocardial region of the left ventricle postinfarction due to marked effects of high shear wall stress in this region, which leads to decreased ATP production and abnormal energy metabolism [244].

Importantly, the decrease in cardiac ATP levels leads to a greater impairment in relaxation, rather than contraction, since many of the transport mechanisms that remove cytosolic calcium following systole are ATP-dependent [245-247]. Therefore, the decrease in ATP and increase in ADP seen in overloaded and failing hearts leads to impaired cardiac diastole as the transport of calcium becomes significantly slower and actin-myosin cross-bridge turnover is impaired [53, 248]. It is important to note that the decrease in cardiac energy levels observed during heart failure are not drastic enough to completely inhibit normal, energy-requiring reactions in the myocytes. This is exemplified by the fact that mice that cannot synthesize Cr still display normal contractility under baseline conditions; however, they display greater susceptibility to I/R injury following treatment with an inotropic agent [249]. As such, though the rate of these reactions is reduced, they can still occur in the failing myocardium, a characteristic that has further detrimental effects due to increased energy consumption by inefficient processes regulating myocardial work, especially those involving the contractile machinery.

In addition, as mentioned previously, the increase in mitochondrial calcium levels contributes to impairments in mitochondrial function. This leads to a decrease in ATP regeneration via uncoupled oxidative phosphorylation, which leads to decreased oxidative capacity and function of the myocardium [250-252]. Compromised

mitochondrial function has further damaging effects because, in addition to reduced glucose oxidation to produce ATP, fatty acid oxidation is also reduced [253, 254]. The latter serves as the main source for ATP in a normal, healthy heart [255] and supplies nearly 70% of total ATP under physiological conditions [256]. Fatty acids utilized for energy by the myocardium are supplied from circulating triglyceride-rich lipoproteins and nonesterified fatty acids, which are metabolized in the mitochondrial matrix to generate acetyl-CoA, the substrate for ATP production via the tricarboxylic acid (TCA) cycle [255].

In heart failure, there is a decrease in fatty acid β-oxidation, which is coupled to an increase in glucose utilization, due to upregulation of enzymes involved in glycolytic pathways [253, 257, 258]. The shift towards glyocolytic metabolism is thought to be a compensatory response in order to maintain myocardial contractility. This is because glucose metabolism leads to a more efficient utilization of oxygen to generate ATP than fatty acid metabolism, which involves mitochondrial ETC uncoupling and can have adverse effects in heart failure [259, 260].

In conjunction with activation of mechanisms that reduce ATP production, energy utilization in remodeled cardiac myocytes is also greatly increased. Cardiomyocyte hypertrophy can further aggravate the imbalance between energy production and utilization. This is especially true in familial hypertrophic cardiomyopathy, where mutations in sarcomeric proteins lead to inefficient ATP utilization and further progression of cardiac dysfunction [261-263].

1.6.5 *Implications of ROS production*

Decreased utilization of fatty acids as a fuel source in heart failure changes the myocardial energetic state. In addition, it also leads to lipid accumulation, and thus cardiac lipotoxicity, due to increased levels of toxic intermediates in the myocardium [264, 265]. These intermediates can lead to impaired cardiac function due to myocardial

atrophy, protein degradation and cell death [266-268]. Lipotoxicity can play a critical role in impaired left ventricular filling and both systolic and diastolic dysfunction [269-271].

It is important to note that mitochondrial uncoupling associated with decreased fatty acid oxidation in heart failure also leads to excessive production of reactive oxygen species (ROS), which is detrimental to cardiac function and can effectively induce cell damage and death [272-274]. In fact, many of the pathways that lead to cardiomyocyte hypertrophy and remodeling following exposure to external stimuli are activated by ROS [275]. In fact, ROS plays a key role in mediating the pathological responses observed Ang II-induced hypertrophy due to its role in activating downstream signaling, including MAPKs [276]. Moreover, ROS appears to be critical for Ang II-mediated hypertrophy as treatment with antioxidants is able to abolish Ang II-induced cardiac remodeling [277].

In addition to its effects on cardiac hypertrophy, ROS can also alter myocardial contractility through a number of different mechanisms. It is able to reduce Ca^{2+} influx via the L-type calcium channels as well as reduce calcium efflux from the cytosol by decreasing the activity of SERCA2a [278, 279]. Furthermore, ROS can reduce myocardial contractility through downstream phosphorylation of troponin T, which reduces calcium sensitivity of the myofilaments as well as through oxidative modification of actin and tropomyosin, which also lead contractile dysfunction [280-283].

Importantly, nearly all of the molecular and cellular abnormalities observed during the progression of cardiac remodeling and heart failure are due to the activation of neurohumoral responses. As mentioned previously, increased sympathetic activity is one of the main neurohumoral mechanisms activated in response to an initial pathological stimulus and alters ventricular hemodynamics to maintain cardiac output at homeostatic levels. Notably, the autonomic nervous system is not comprised solely of the sympathetic nervous system. Rather, parasympathetic signaling in the periphery is able to oppose many of the actions of adrenergic signaling, including the positive chronotropic and inotropic effects observed in heart failure during a state of enhanced sympathetic

signaling. As such, it is reasonable to expect changes in cholinergic signaling coupled with altered sympathetic tone in the failing heart.

1.7 Regulation of cholinergic signaling

ACh is the major chemical neurotransmitter in the periphery and regulates sympathetic tone through signaling at pre-ganglionic sites and parasympathetic tone via signaling at both pre- and post-ganglionic nerve terminals. The mechanisms regulating ACh production and secretion are well known. Genesis of ACh depends on the actions of the enzyme choline acetyltransferase (ChAT) [284] which converts free choline and acetyl-CoA into ACh, a process first described by Nachmansohn and Machado [285]. ChAT is in kinetic excess in nerve terminals; therefore, minor reductions in ChAT activity have negligible impact on ACh content and release [286]. ACh synthesis is exquisitely coupled to the high-affinity choline transporter (CHT1), which serves as the rate-limiting step for ACh production [287]. This is due to the importance of CHT1 in regulating the transport of sufficient amounts of free choline from the extracellular environment into the presynaptic nerve terminal [288] where it can be utilized to synthesize ACh and maintain its sustained release. No other choline transporter can replace CHT1, as its genetic deletion leads to death shortly after birth due to respiratory failure [289]. The secretion of ACh into the extracellular environment is dependent on its packaging into exocytic vesicles via the vesicular acetylcholine transporter (VAChT)[290]. The secretory vesicle requires two distinct components for the proper packaging of ACh into the exocytic vesicles. Firstly, a vacuolar type H^+-ATPase is required to transfer protons from the cytoplasm into the vesicle. Secondly, the VAChT, a 12-transmembrane domain protein, is essential as it utilizes the electrochemical gradient generated by the proton ATPase to store ACh in synaptic vesicles [290].

As part of the same family of transporters as VMAT1 and VMAT2, which function to transport monamines, including dopamine and norepinephrine, into synaptic vesicles, VAChT acts in a similar manner as a proton/amine antiporter [291, 292]. This transporter

is present in lower species, such as *Caenorhabditis elegans*, where the vesicular acetylcholine transporter gene (*unc-17*) appears to have a functional role as mutations in *unc-17* protect against organophosphorous toxicity in nematodes [293]. Normally, organophosphorous compounds induce toxicity by inhibiting ACh degradation in the synaptic cleft and thereby maintaining high levels of extracellular ACh. Decreased function of *unc-17* decreases ACh secretion and thereby protects against toxicity, thus indicating a role for this transporter in cholinergic signaling. In addition, mammalian species, including rats and humans, also express VAChT [290, 294], suggesting a conserved functional role for this transporter in mediating ACh packaging in secretory vesicles across species.

Interestingly, the VAChT gene is part of the "cholinergic gene locus," a unique arrangement in the mammalian genome. This locus is comprised of both the ChAT and VAChT genes as well as the regulatory elements modulating gene transcription in this locus [295]. In both *Drosophila* as well as mammals, the entire VAChT coding sequence is found within the first intron of the ChAT gene [290, 295, 296]. This genetic arrangement allows for a precise and intricate regulation of cholinergic neurotransmission and, therefore, complex downstream functions in both the central nervous system as well as the parasympathetic system.

Cholinergic neurotransmission is critically dependent on the function of VAChT, as inhibition of this transporter using vesamicol decreases ACh packaging into synaptic vesicles and leads to inhibition of ACh secretion [297]. Furthermore, elimination of the VAChT gene abolishes stimulated ACh release [298]. In addition, decreased expression levels of VAChT lead to proportional decreases in ACh release [299]. Overexpression of VAChT in immature *Xenopus* neurons leads to increased synaptic responses [300]. Furthermore, overexpression of VAChT in mice has been shown to increase ACh release [301, 302], improve physical fitness and cause abnormalities in cognitive behavior [301]. Hence, VAChT is unique in its ability to regulate ACh release.

ACh can bind five different muscarinic receptor subtypes in the body; however, in the cardiovascular system, the M_2 and M_3 subtypes play critical roles in regulating cholinergic signaling. These actions are mediated by the binding of ACh to M_2 muscarinic receptors in the atrial myocytes [303, 304]. M_2 receptors are coupled to G_i proteins, which mediate the decrease in inotropic and chronotropic responses following muscarinic receptor activation through various downstream mechanisms. One involves direct inhibition of adenylyl cyclase, which leads to decreased production of cAMP and activation of protein kinase A (PKA) [305, 306]. Muscarinic receptor activation can also lead to indirect inhibition of L-type Ca^{2+} channels through a decrease in cAMP production [307]. Furthermore, the negative inotropic and chonotropic effects observed following activation of M_2 receptors is due to hyperpolarization of atrial cells following the direct activation of inwardly rectifying ACh sensitive potassium channels (K_{ACh}) by the $G_i\beta\gamma$ subunit of the M_2 receptor [308]. In addition, the $G_i\beta\gamma$ subunit is also able to activate NO production through PI3K-Akt mediated phosphorylation of eNOS.

Although the M_2 receptor is the main muscarinic subtype in the heart, it has previously been shown that M_3 muscarinic receptors also play a role in mediating physiological signaling in the heart. Previous work confirmed the importance of cholinergic signaling via the M_3 receptor subtype as pre-treatment with an M_3-selective inhibitor could attenuate the increase in IP_3 formation observed following treatment with carbachol [309]. This study suggests that cholinergic signaling via the M_3 muscarinic receptor is coupled to G_q-mediated activation of the PLC/IP_3 pathway. Furthermore, M_3 receptors also mediate inotropic signaling in the atria as the biphasic inotropic response observed in isolated atria can be attributed to both M_2 and M_3 receptor signaling, with the latter mediating the positive inotropic response [310]. M_3 muscarinic receptors also appear to regulate pathophysiological responses. It has previously been reported that cholinergic signaling via M_3 receptors in the heart can lead to cardioprotection following myocardial ischemia, a process thought to occur through inhibition of miR-376b-5p [311]. Furthermore, it was recently shown that upregulation of the M_3 muscarinic receptor in mice attenuates angiotensin II-induced cardiac hypertrophic response [312].

ACh has also been shown to bind to M_3 muscarinic receptors in the vascular smooth muscles cells and thereby induce vasoconstriction via activation of phospholipase C and IP_3 production. However, these responses are typically ineffective under physiological conditions as ACh can bind to endothelial cells in the vasculature and induce secretion of nitric oxide (NO), which acts as a potent vasodilator in the cardiovascular system.

In addition to the direct effects of ACh on the heart via muscarinic receptors, it can also regulate sympathetic signaling by binding to presynaptic receptors on sympathetic nerve terminals innervating the heart. Binding of ACh to M_1 muscarinic receptors at presynaptic sympathetic nerves can increase NE release [313], while M_2 muscarinic receptors appear to mediate ACh-induced inhibition of NE release at the atria [314, 315]. GPCR signaling via downstream G proteins is partially modulated by the actions of the Regulator of G Protein Signaling (RGS) proteins, which can terminate G protein signaling by increasing hydrolysis of bound GTP [316]. RGS2, in particular, has been shown to decrease both G_q and G_i protein signaling [317-319], though it shows G_q-selectivity and acts on the latter with a lower potency [320]. In addition, RGS2 has previously been shown to reduce adenylyl cyclase activity downstream of G_s signaling [321]. Importantly, RGS2 can inhibit hypertrophy in response to β-adrenergic stimulation [322], an effect that may partially be due to its role in inhibiting eIF2B-mediated protein synthesis [323]. Furthermore, RGS2 KO mice display hypertension under basal conditions [324] and increased ventricular dysfunction and dilation following transverse aortic constriction (TAC) [325]. These data suggest that RGS2 signaling plays a role in regulating signaling through both muscarinic and adrenergic receptors and modulating the cardiovascular response to pathological stimuli by altering autonomic regulation of the heart.

1.8 Parasympathetic hypoactivity in heart failure

Hyperactivity of the sympathetic nervous system in heart failure is coupled with decreased activation of the parasympathetic nervous system leading to decreased signaling through ACh [326]. A decrease in parasympathetic signaling is observed early following induction of cardiac remodeling even prior to the onset of heart failure, and this cholinergic hypoactivity appears to contribute to the cardiac dysfunction observed in HF [326]. The importance of cholinergic signaling in cardiac remodeling is further highlighted by previous data suggesting a strong association between decreased vagal reflex, as measured through baroreceptor signaling, and ventricular arrhythmias following myocardial infarction [327, 328]. This decrease in cholinergic signaling may partially be due to the production of autoantibodies against M_2-muscarinic receptors, which significantly reduce ligand binding in patients with idiopathic dilated cardiomyopathy and effectively inhibit downstream signaling [329].

Importantly, it was previously reported that pacing-induced LV dysfunction and heart failure in dogs leads to changes in both sympathetic as well as vagal control of the heart [330]. Furthermore, changes in vagus nerve-mediated regulation of heart rate have been observed during the early stages of LV dysfunction, suggesting that alterations in cholinergic signaling in the heart may contribute to the HF phenotype [330]. These changes in vagal control of heart rate appear to occur to both cardiac post-ganglionic signaling, as well as alterations in parasympathetic pre-ganglionic signaling [331].

In agreement with the notion of a causal relationship between lower cholinergic signaling and cardiac dysfunction, M_2 muscarinic receptor knockout mice exhibit a greater impairment in ventricular function following an acute dose of phenylephrine, which increases cardiac afterload [332]. Although these mice exhibited no difference in ventricular function under baseline conditions, chronic treatment with isoproterenol to induce cardiac remodeling led to a significant increase in matrix metalloproteinase (MMP) activity in M_2 receptor KO mice, as compared to WT mice [332]. This suggests that cholinergic tone may play a role in regulating MMP activity and, therefore, the progression of cardiac dysfunction as increased MMP activity has been associated with ventricular dysfunction in patients with heart failure [35, 333]. In addition, we have

previously reported that mice with reduced cholinergic neurotransmission due to decreased expression of VAChT exhibit cardiac remodeling and dysfunction [334]. It has also been reported that mice with a decrease in the expression of the presynaptic, high affinity choline transporter (CHT1; CHT1 heterozygous), a protein necessary for the uptake of choline and thus crucial for ACh synthesis, leads to age-dependent ventricular remodeling and dysfunction [335].

There are several possible mechanisms that may lead to decreased cholinergic signaling in the heart. One such mechanism may involve the activation of mechanoreceptors in dilated hearts, due to greater mechanical stretch of the cardiac muscle. This mechanical stretch activates sympathetic afferent fibres which, in turn, leads to a reflex inhibition of intracardiac cholinergic signaling [336]. Due to the decrease in parasympathetic signaling observed in heart failure, increasing cholinergic tone may serve to improve outcomes in HF patients.

1.9 Parasympathetic system as a target in the failing heart

Both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) modulate extracellular levels of ACh by regulating its hydrolysis. In fact, it has been shown that AChE KO mice are able to utilize BChE to break down acetylcholine, suggesting a role for this enzyme in the regulation of cholinergic neurotransmission [337]. Although the heart contains both AChE and BChE, the levels of BChE are much higher than those of AChE [338, 339]. Furthermore, both AChE and BChE are found in blood; however, the levels of circulating BChE are significantly greater than those of AChE [338] and likely serve to regulate ACh metabolism throughout the body. A previous report has suggested an inverse association between serum levels of butyrylcholinesterase (BuChE) and overall mortality in middle-aged and elderly individuals [340]. Furthermore, BuChE levels have been associated with several cardiovascular risk factors including albumin, cholesterol and triglycerides [340]. An additional study suggested that increasing levels of BuChE were associated with negative long-term outcomes, including mortality, in

patients with stable coronary artery disease (CAD) [341]. In fact, the same group of researchers utilized cholinesterase activity as a biomarker to predict mortality in CAD patients and found a very strong inverse relationship between cholinesterase activity and prediction of mortality [342].

Although the aforementioned studies suggest the need for at least a minimal level of cholinesterase activity to reduce mortality in patients with cardiovascular disease, accumulating evidence suggests that restoring cholinergic activity after experimental heart failure can improve functional outcomes. In fact, in an animal model of heart failure, the addition of vagal nerve stimulation to β-blockade therapy led to improvements in cardiac contractility, as measured through LV systolic and end-diastolic pressure, as well as animal survival [343, 344]. Additionally, chronic treatment with the cholinesterase inhibitor, donepezil, reduces cardiac remodeling and increases survival rates in animal models of heart failure [345, 346]. Furthermore, increasing ACh levels through administration of a peripheral cholinesterase inhibitor, pyridostigmine, led to greater vagal control of the heart and a reduction in ventricular dysfunction associated with heart failure in rats [347]. Importantly, the positive effect of enhancing cholinergic tone is not limited to animal models of heart failure. A clinical trial in which a small group of patients were subjected to vagal stimulation via an implantable system and followed for up to 6 months showed an increase in both their NYHA class score as well as LV end-systolic volume [348]. Furthermore, it has been shown that vagal nerve stimulation is tolerated well and improves quality of life and LV function in chronic heart failure patients [349]. Additionally, it was recently reported that the use of cholinesterase inhibitors in patients with Alzheimer's disease led to a 34% reduction in risk of myocardial infarction and death [350]. Furthermore, a Phase II clinical trial is currently underway to study the safety of the cholinesterase inhibitor, pyridostigmine, when administered alone in HF patients (Clinicaltrials.gov Identifier: NCT01415921). This study may serve as a precursor for additional clinical trials aimed at developing a novel therapeutic approach for the treatment of HF.

The mechanisms of action leading to the protective effects of cholinesterase inhibition have yet to be completely understood. One possibility is that cholinesterase inhibitors can reduce remodeling and cardiac dysfunction by directly enhancing the levels of ACh. However, it is also possible that quarternary cholinesterase inhibitors, such as pyridostigmine, can impact the progression of cardiac dysfunction through an indirect pathway via their ability to alter gene expression, as previously shown in the brain [351, 352]. Although this phenomenon has not yet been reported in the heart, it is possible that a similar mechanism of action as that observed in the brain also exists in cardiac tissue and contributes to reduced remodeling and dysfunction in heart failure.

Increasing systemic ACh levels through the use of cholinesterase inhibitors appears to decrease dysfunction following induction of cardiac disease. Additionally, oral administration of choline also appears to decrease the hypertrophic response in mice subjected to transverse aortic constriction (TAC), an effect that may be dependent on the actions of a specific miRNA, miR-133a, as well as calcineurin [353]. Importantly, the anti-hypertrophic effects of orally administered choline are due to its direct actions on cardiomyocytes, as choline treatment is able to prevent cellular hypertrophy in cultured cardiomyocytes treated with isoproterenol in order to induce remodeling [353].

The effect of choline is not limited to protection against afterload-induced hypertrophy. It was previously reported that choline pre-treatment also leads to a reduction in Ang II-induced remodeling through inhibition of cellular signaling via the ROS-mediated p38 MAPK pathway as well as the Ca^{2+}-calcineurin signaling pathway, both of which are associated with Ang II-mediated cardiac dysfunction [354]. The protective effect of choline appears to be due to its direct actions on cardiomyocytes as pre-treatment was also able to attenuate the Ang II-induced hypertrophic response in cultured neonatal cardiomyocytes [354]. Although choline treatment appears to have a protective effect, it is yet unclear whether this is due to its role in the production of ACh or due to the fact that choline can act as a direct agonist of M_3 muscarinic receptors, which have been linked to cardioprotection.

In agreement with the notion that cholinergic tone in the heart plays a role in HF, cardiac sympathetic nerves switch neurotransmitters and undergo cholinergic transdifferentiation during heart failure in both animal models and humans [355]. Genetic inhibition of this transdifferentiation increases mortality of animal models suggesting that this transdifferential switch is protective [355]. This observation agrees with previous findings from our laboratory that genetic disturbance of cholinergic machinery in mice leads to cardiac dysfunction, a phenotype that can be reversed by treatment with peripheral cholinesterase inhibitors [334].

1.10 Non-neuronal cholinergic system

For many years, ACh has been classified as the major neurotransmitter for parasympathetic signaling. The importance of ACh in the nervous system is indisputable; however, ACh can also play a significant role in cells other than neurons as the non-neuronal cholinergic system (NNCS) appears to be linked to proper function of various organ systems in both health and disease. ACh is a phylogenetically ancient molecule that can be produced and secreted by both prokaryotes (bacteria) and lower order eukaryotes including fungi and protozoa [284]. Furthermore, several different cell types in higher order eukaryotes, including humans, utilize ACh as a signaling molecule. These include keratinocytes, which have been shown to produce ACh at very high levels [356], as well as both oesophageal and upper respiratory tract epithelial cells [357, 358]. Furthermore, prototypical markers of the cholinergic system have also been detected in the kidney [359], as well as pancreatic α-cells where non-neuronal ACh plays a crucial role in priming β-cells to secrete insulin in humans [360]. Finally, the NNCS is active in regulating immune function as T-cells secrete ACh, which can then act in an autocrine/paracrine fashion to inhibit inflammatory cytokine production [361, 362].

Increasing systemic cholinergic tone leads to positive outcomes with regards to left ventricular remodeling and function in various HF models. A universally accepted

principle in autonomic regulation of cardiac function is that parasympathetic innervation of the ventricles is very sparse [363, 364]. However, vagal stimulation has been shown to reduce ventricular contractility in the absence of sympathetic drive [365-367]. These direct negative inotropic effects in response to vagal stimulation have led to further analysis of parasympathetic innervation in the ventricles. Some studies suggest that, contrary to previous work, cholinergic nerve fibers are abundantly present in the ventricles. Immunostaining for AChE has revealed a dense innervation of both the endocardial and epicardial surfaces of the ventricles in several species, including human [368-370]. Additionally, immunostaining using the cholinergic marker, CHT1, has revealed that the presence of parasympathetic nerve fibers in the ventricles is mainly restricted to the cardiac conduction system, although some staining was observed in the ventricular free wall [363]. In addition to vagal innervation of the ventricles, it has recently been proposed that cardiomyocytes are also able to synthesize and release ACh at the cellular level. Cardiomyocytes possess the machinery (ChAT, VAChT and CHT1) for *de novo* production and storage of ACh [371, 372]. It has been suggested that ventricular cardiomyocytes may synthesize ACh. This non-neuronal ACh may then act in an autocrine/paracrine fashion to amplify neuronal cholinergic signaling and thereby protect the heart under stressful conditions, including heart failure [372]. VAChT, which is an important component of the cholinergic machinery, was shown to be present in vesicles in cardiomyocytes [371] suggesting a quantal release of ACh from these cells.

Other studies have also recently highlighted the importance of this non-neuronal cholinergic system. Previous work has shown that ChAT KO HL-1 cells, derived from murine atrial cardiac tissue, display lower levels of cellular ATP, which leads to decreased viability following induction of chemical hypoxia [375]. In addition, ChAT KO HL-1 cells appear to generate greater levels of ROS than control cells following an acute challenge with norepinephrine, as measured using the ROS indicator APF [375]. This increase in ROS production in ChAT KO cells may contribute to the increased caspase-3 activation and apoptotic response that was observed following norepinephrine treatment [375]. Conversely, cardiac-specific overexpression of choline acetyltransferase (ChAT-Tg), the enzyme responsible for the synthesis of ACh, appears to decrease

remodeling and enhance survival following myocardial infarction [376]. These ChAT-Tg mice also appear to be less susceptible to *ex vivo* ischemia-reperfusion injury, which was induced using a Langerdorff apparatus [376]. The infarcted region was significantly decreased in the ChAT-Tg mice and the time from onset of ischemia to beating arrest was significantly increased in the mutant mice [376]. These data further highlight the importance of the cardiac non-neuronal cholinergic system following induction of stress, including ischemia. Together, these data provide for an unanticipated mechanism by which non-neuronal ACh may play an important role in cardiac function.

1.11 Cholinergic anti-inflammatory pathway

ACh secretion from non-neuronal cells is not unique to cardiomyocytes. In fact, lymphocytes can also release ACh, which plays a crucial role in regulating the immune response by activating an inflammatory reflex, the cholinergic anti-inflammatory pathway [361]. This reflex is dependent on the peripheral actions of ACh released from the Vagus [377, 378]; however, nerve fibers innervating the splenic nerve from the celiac ganglion are adrenergic, rather than cholinergic [379]. As such, the presence of ACh in the spleen is not due to neuronal cholinergic innervation but, rather, activation of the efferent arc of the Vagus nerve and downstream activation of splenic T cells, which then secrete ACh [361]. This T cell-derived ACh can then activate α7 nAChRs on macrophages and thereby inhibiting pro-inflammatory cytokine secretion including TNFα, IL-1β and IL-6 [377, 378]. The anti-inflammatory effects of ACh appear to be mediated specifically by its interaction with the α7 receptors on macrophages as this effect can be blunted using selective nicotinic antagonists or α7 receptor knockout mice [378, 380-382].

This pathway appears to play a crucial role in the progression of several cardiovascular diseases. In fact, in a two-kidney, one-clip animal model of hypertension, a significant downregulation of α7 receptors, coupled with an increase in serum levels of TNFα, was

previously observed [383]. This occurred downstream of reduced vagal tone thus suggesting that the cholinergic anti-inflammatory pathway plays a role in the induction of secondary hypertension.

Increased expression of inflammatory cytokines, which may serve as an indicator of reduced activity of the cholinergic anti-inflammatory reflex, has been observed in both systemic circulation during chronic heart failure as well as in the failing myocardium [384, 385]. Activation of the cholinergic anti-inflammatory pathway, which is dependent on both vagal as well as non-neuronal ACh, has been shown to be beneficial in heart failure [386], and may contribute to the cardioprotective effects of increased cholinergic tone observed in several animal models of heart failure, including volume overload, tachycardia and myocardial infarction [343-346]. This cholinergic anti-inflammatory pathway serves as an important determinant of the extent of cardiac remodeling following induction of cardiac disease. Notably, it appears to be altered in heart failure due to the decrease in parasympathetic tone, and thus peripheral ACh secretion. In fact, it was recently shown that, following LPS-induced endotoxemia in rats, vagal nerve stimulation is able to partially attenuate myocardial inflammation and hemodynamic alterations [387]. Furthermore, the inflammatory response in these rats was enhanced following vagotomy, thus suggesting that parasympathetic signaling via the Vagus plays a critical role in reducing the local inflammatory response and decreasing cardiac dysfunction [387].

Although it is still unclear whether there is a direct correlation between the levels of circulating inflammatory cytokines and cardiac dysfunction, recent observations suggest a possible relationship between inflammation and progression of heart failure. In a canine high-rate pacing model of heart failure, chronic vagal nerve stimulation led to significant inhibition of heart failure development, which was associated with attenuated systemic inflammation [344], thus suggesting a possible beneficial effect of reduced inflammatory cytokines in heart failure. Additionally, spontaneously hypertensive rats (SHRs), as well as those with abdominal aorta coarctation-induced hypertension, exhibited greater end organ damage (EOD) coupled with decreased levels of the α7nAChR [388]. Furthermore, chronic treatment of SHRs with an α7nAChR agonist led to decreased tissue levels of

pro-inflammatory cytokines which was associated with decreased EOD [388]. Increased levels of inflammatory cytokines have been shown to be directly related to decreased left ventricular ejection fraction [389]. Moreover, increased levels of the pro-inflammatory cytokine TNFα can induce cardiac hypertrophy and promote cardiomyocyte apoptosis, thus contributing to the onset and progression of ventricular dysfunction and remodeling [384, 390]. These data suggest an important role for the cholinergic inflammatory reflex in regulating cardiac function and attenuating the progression of heart failure, perhaps by regulating circulating cytokines such as TNFα.

Despite the overwhelming evidence for immune regulation through peripheral cholinergic signaling, it was recently reported that the reflex activation of the inflammatory system by the autonomic nervous system following LPS treatment is due to the actions of the sympathetic system, and not the Vagus nerve [391]. However, contrary to this recently published report, it is well established that the parasympathetic nervous system is very important in regulating the innate immune response and can inhibit cytokine release and systemic inflammation [392]. In fact, several studies have provided novel insight into the specific mechanisms through which neuronal ACh can act peripherally to control the immune response and regulate the extent of cardiac dysfunction.

1.12 Rationale and Hypothesis

Heart failure is a very prominent progressive clinical syndrome and is exemplified by a number of structural and molecular changes, which take place following activation of neurohumoral mechanisms to maintain cardiac homeostasis. Enhanced sympathetic signaling is a critical modulator of neurohumoral signaling, but it can have severe adverse effects due to long-term activation. Conversely, it is abundantly clear that increased sympathetic activation is often coupled with reduced parasympathetic signaling in heart failure. However, the role of the parasympathetic system in cardiac dysfunction has not

been thoroughly studied, despite its importance in regulating various physiological pathways (Fig. 1.3). As such, enhanced cholinergic signaling has yet to be utilized clinically as a pharmacological target, partly due to the lack of information available with regards to the specific changes that take place solely due to reduced cholinergic signaling in heart failure. Therefore, it is important to further our understanding of the mechanisms through which cholinergic signaling can modulate cardiac function. In addition, the role of parasympathetic signaling in cardiac disease must be elucidated to determine its contribution to abnormal signaling in the remodeled myocardium.

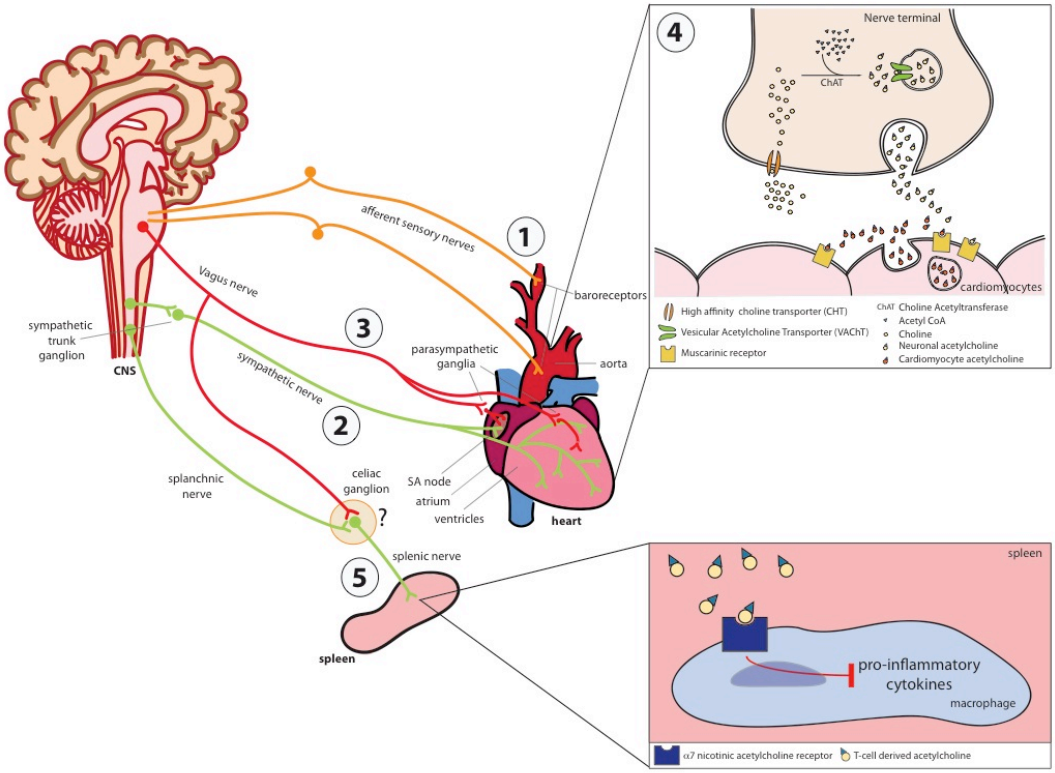
Therefore, the overall objective of this thesis is to determine whether altered cholinergic signaling plays a role in regulating cardiac function under physiological conditions as well as whether altered cholinergic signaling can influence the progression of cardiac dysfunction. Specifically, the aim of this thesis is to:

1. Examine the molecular alterations that contribute to cardiac dysfunction in a mouse model with deficient cholinergic signaling.
2. Determine whether the non-neuronal cholinergic system plays a role in regulating cardiac function and ventricular contractility, under both physiological and pathological conditions.
3. Determine whether enhanced cholinergic signaling influences cardiac function under physiological conditions.

Previously, several lines of research have begun to highlight the importance of cholinergic signaling in maintaining cardiovascular homeostasis in both health and disease. As such, *we hypothesized that alterations in both neuronal and non-neuronal cholinergic signaling can regulate cardiac function under baseline conditions as well as modify the progression of cardiac remodeling, a precursor to the onset of heart failure*.

Figure 1.3 - Autonomic regulation of cardiac function. (1) Baroreceptors in the carotid arteries and aortic arch monitor arterial pressure. Increased arterial pressure activates signaling to the Nucleus of the Tractus Solitarius (NTS) and reduces sympathetic output and simultaneously activates the parasympathetic nervous system. A decrease in arterial pressure has the opposite effect. **(2)** Activation of sympathetic nerves leads to positive chronotropic and inotropic responses as well as arteriolar constriction. **(3)** Activation of the parasympathetic system via the vagus nerve leads to negative chronotropy and lusitropy. **(4)** The cardiac non-neuronal cholinergic system amplifies parasympathetic cholinergic signaling and regulates heart rate recovery following activity. In addition, the cardiomyocyte-derived ACh prevents cardiac remodeling and hypertrophy under physiological conditions. **(5)** Vagus and/or sympathetic splanchnic nerve stimulation leads to activation of the anti-inflammatory pathway, which involves secretion of non-neuronal ACh from splenic T cells. This T cell-derived ACh activates α7 nicotinic acetylcholine receptors (α7nAChRs) on macrophages and inhibits the release of pro-inflammatory cytokines.

Figure 1.3 - Autonomic regulation of cardiac function.



1.13 References

[1] Kent KM, Epstein SE, Cooper T, Jacobowitz DM. Cholinergic innervation of the canine and human ventricular conducting system. Anatomic and electrophysiologic correlations. Circulation. 1974;50:948-55.

[2] Just A, Faulhaber J, Ehmke H. Autonomic cardiovascular control in conscious mice. American journal of physiology Regulatory, integrative and comparative physiology. 2000;279:R2214-21.

[3] Olshansky B, Sabbah HN, Hauptman PJ, Colucci WS. Parasympathetic nervous system and heart failure: pathophysiology and potential implications for therapy. Circulation. 2008;118:863-71.

[4] Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation. 2014;129:e28-e292.

[5] Hosenpud JD, Stibolt TA, Atwal K, Shelley D. Abnormal pulmonary function specifically related to congestive heart failure: comparison of patients before and after cardiac transplantation. The American journal of medicine. 1990;88:493-6.

[6] Snashall PD, Chung KF. Airway obstruction and bronchial hyperresponsiveness in left ventricular failure and mitral stenosis. The American review of respiratory disease. 1991;144:945-56.

[7] Kraemer MD, Kubo SH, Rector TS, Brunsvold N, Bank AJ. Pulmonary and peripheral vascular factors are important determinants of peak exercise oxygen uptake in patients with heart failure. J Am Coll Cardiol. 1993;21:641-8.

[8] Puri S, Baker BL, Dutka DP, Oakley CM, Hughes JM, Cleland JG. Reduced alveolar-capillary membrane diffusing capacity in chronic heart failure. Its pathophysiological relevance and relationship to exercise performance. Circulation. 1995;91:2769-74.

[9] Guazzi M, Pontone G, Brambilla R, Agostoni P, Reina G. Alveolar--capillary membrane gas conductance: a novel prognostic indicator in chronic heart failure. European heart journal. 2002;23:467-76.

[10] Stead EA, Jr. Renal factor in congestive heart failure. Circulation. 1951;3:294-9.

[11] Levy MN, Martin PJ, Stuesse SL. Neural regulation of the heart beat. Annual review of physiology. 1981;43:443-53.

[12] Veeraraghavan R, Gourdie RG, Poelzing S. Mechanisms of cardiac conduction: a history of revisions. American journal of physiology Heart and circulatory physiology. 2014;306:H619-27.

[13] Limas CJ, Olivari MT, Goldenberg IF, Levine TB, Benditt DG, Simon A. Calcium uptake by cardiac sarcoplasmic reticulum in human dilated cardiomyopathy. Cardiovasc Res. 1987;21:601-5.

[14] Takeishi Y, Chu G, Kirkpatrick DM, Li Z, Wakasaki H, Kranias EG, et al. In vivo phosphorylation of cardiac troponin I by protein kinase Cbeta2 decreases cardiomyocyte calcium responsiveness and contractility in transgenic mouse hearts. The Journal of clinical investigation. 1998;102:72-8.

[15] El-Armouche A, Pohlmann L, Schlossarek S, Starbatty J, Yeh YH, Nattel S, et al. Decreased phosphorylation levels of cardiac myosin-binding protein-C in human and experimental heart failure. Journal of molecular and cellular cardiology. 2007;43:223-9.

[16] Sequeira V, Nijenkamp LL, Regan JA, van der Velden J. The physiological role of cardiac cytoskeleton and its alterations in heart failure. Biochimica et biophysica acta. 2014;1838:700-22.

[17] Lin M, Sumimoto T, Hiwada K. Left ventricular geometry and cardiac function in mild to moderate essential hypertension. Hypertension research : official journal of the Japanese Society of Hypertension. 1995;18:151-7.

[18] Mangano DT, Van Dyke DC, Ellis RJ. The effect of increasing preload on ventricular output and ejection in man. Limitations of the Frank-Starling Mechanism. Circulation. 1980;62:535-41.

[19] Gleason WL, Braunwald E. Studies on Starling's law of the heart. VI. Relationships between left ventricular enddiatolic volume and stroke volume in man with observations on the mechanism of pulsus alternans. Circulation. 1962;25:841-8.

[20] Kober L, Torp-Pedersen C, Pedersen OD, Hoiberg S, Camm AJ. Importance of congestive heart failure and interaction of congestive heart failure and left ventricular systolic function on prognosis in patients with acute myocardial infarction. The American journal of cardiology. 1996;78:1124-8.

[21] Braunwald E, Pfeffer MA. Ventricular enlargement and remodeling following acute myocardial infarction: mechanisms and management. The American journal of cardiology. 1991;68:1D-6D.

[22] Hunter JJ, Chien KR. Signaling pathways for cardiac hypertrophy and failure. The New England journal of medicine. 1999;341:1276-83.

[23] Velagaleti RS, Gona P, Pencina MJ, Aragam J, Wang TJ, Levy D, et al. Left ventricular hypertrophy patterns and incidence of heart failure with preserved versus reduced ejection fraction. The American journal of cardiology. 2014;113:117-22.

[24] Drazner MH, Rame JE, Marino EK, Gottdiener JS, Kitzman DW, Gardin JM, et al. Increased left ventricular mass is a risk factor for the development of a depressed left ventricular ejection fraction within five years: the Cardiovascular Health Study. J Am Coll Cardiol. 2004;43:2207-15.

[25] Grogan M, Redfield MM, Bailey KR, Reeder GS, Gersh BJ, Edwards WD, et al. Long-term outcome of patients with biopsy-proved myocarditis: comparison with idiopathic dilated cardiomyopathy. J Am Coll Cardiol. 1995;26:80-4.

[26] Cooper LT, Jr. Myocarditis. The New England journal of medicine. 2009;360:1526-38.

[27] van Heerebeek L, Borbely A, Niessen HW, Bronzwaer JG, van der Velden J, Stienen GJ, et al. Myocardial structure and function differ in systolic and diastolic heart failure. Circulation. 2006;113:1966-73.

[28] Wakatsuki T, Schlessinger J, Elson EL. The biochemical response of the heart to hypertension and exercise. Trends in biochemical sciences. 2004;29:609-17.

[29] Grossman W. Diastolic dysfunction in congestive heart failure. The New England journal of medicine. 1991;325:1557-64.

[30] Baicu CF, Zile MR, Aurigemma GP, Gaasch WH. Left ventricular systolic performance, function, and contractility in patients with diastolic heart failure. Circulation. 2005;111:2306-12.

[31] Zile MR, Baicu CF, Gaasch WH. Diastolic heart failure--abnormalities in active relaxation and passive stiffness of the left ventricle. The New England journal of medicine. 2004;350:1953-9.

[32] Banerjee P, Banerjee T, Khand A, Clark AL, Cleland JG. Diastolic heart failure: neglected or misdiagnosed? J Am Coll Cardiol. 2002;39:138-41.

[33] Brutsaert DL, Sys SU, Gillebert TC. Diastolic failure: pathophysiology and therapeutic implications. J Am Coll Cardiol. 1993;22:318-25.

[34] Ruzumna P, Gheorghiade M, Bonow RO. Mechanisms and management of heart failure due to diastolic dysfunction. Curr Opin Cardiol. 1996;11:269-75.

[35] Martos R, Baugh J, Ledwidge M, O'Loughlin C, Conlon C, Patle A, et al. Diastolic heart failure: evidence of increased myocardial collagen turnover linked to diastolic dysfunction. Circulation. 2007;115:888-95.

[36] Lindsay MM, Maxwell P, Dunn FG. TIMP-1: a marker of left ventricular diastolic dysfunction and fibrosis in hypertension. Hypertension. 2002;40:136-41.

[37] Melendez GC, McLarty JL, Levick SP, Du Y, Janicki JS, Brower GL. Interleukin 6 mediates myocardial fibrosis, concentric hypertrophy, and diastolic dysfunction in rats. Hypertension. 2010;56:225-31.

[38] Rosen BD, Edvardsen T, Lai S, Castillo E, Pan L, Jerosch-Herold M, et al. Left ventricular concentric remodeling is associated with decreased global and regional systolic function: the Multi-Ethnic Study of Atherosclerosis. Circulation. 2005;112:984-91.

[39] Schrier RW. Body fluid volume regulation in health and disease: a unifying hypothesis. Annals of internal medicine. 1990;113:155-9.

[40] Schrier RW. Pathogenesis of sodium and water retention in high-output and low-output cardiac failure, nephrotic syndrome, cirrhosis, and pregnancy (2). The New England journal of medicine. 1988;319:1127-34.

[41] Rowell LB, O'Leary DS. Reflex control of the circulation during exercise: chemoreflexes and mechanoreflexes. Journal of applied physiology. 1990;69:407-18.

[42] Corcoran AC, Page IH. Effects of Hypotension Due to Hemorrhage and of Blood Transfusion on Renal Function in Dogs. The Journal of experimental medicine. 1943;78:205-24.

[43] Anker SD, von Haehling S. Inflammatory mediators in chronic heart failure: an overview. Heart. 2004;90:464-70.

[44] Hofmann U, Frantz S. How can we cure a heart "in flame"? A translational view on inflammation in heart failure. Basic Res Cardiol. 2013;108:356.

[45] Goldstein RE, Beiser GD, Stampfer M, Epstein SE. Impairment of autonomically mediated heart rate control in patients with cardiac dysfunction. Circulation research. 1975;36:571-8.

[46] Eaton GM, Cody RJ, Nunziata E, Binkley PF. Early left ventricular dysfunction elicits activation of sympathetic drive and attenuation of parasympathetic tone in the paced canine model of congestive heart failure. Circulation. 1995;92:555-61.

[47] Ma SX, Long JP. Positive chronotropic and inotropic responses to release of norepinephrine from sympathetic nerve terminals produced by nitroglycerin in atria. Archives internationales de pharmacodynamie et de therapie. 1991;309:125-36.

[48] Furnival CM, Linden RJ, Snow HM. The inotropic and chronotropic effects of catecholamines on the dog heart. The Journal of physiology. 1971;214:15-28.

[49] Allen IS, Cohen NM, Dhallan RS, Gaa ST, Lederer WJ, Rogers TB. Angiotensin II increases spontaneous contractile frequency and stimulates calcium current in cultured neonatal rat heart myocytes: insights into the underlying biochemical mechanisms. Circulation research. 1988;62:524-34.

[50] Walker BR, Childs ME, Adams EM. Direct cardiac effects of vasopressin: role of V1- and V2-vasopressinergic receptors. The American journal of physiology. 1988;255:H261-5.

[51] Moravec CS, Reynolds EE, Stewart RW, Bond M. Endothelin is a positive inotropic agent in human and rat heart in vitro. Biochemical and biophysical research communications. 1989;159:14-8.

[52] Chaudhri B, Del Monte F, Hajjar RJ, Harding SE. Interaction between increased SERCA2a activity and beta -adrenoceptor stimulation in adult rabbit myocytes. American journal of physiology Heart and circulatory physiology. 2002;283:H2450-7.

[53] Tian R, Nascimben L, Ingwall JS, Lorell BH. Failure to maintain a low ADP concentration impairs diastolic function in hypertrophied rat hearts. Circulation. 1997;96:1313-9.

[54] Horn M, Remkes H, Stromer H, Dienesch C, Neubauer S. Chronic phosphocreatine depletion by the creatine analogue beta-guanidinopropionate is associated with increased

mortality and loss of ATP in rats after myocardial infarction. Circulation. 2001;104:1844-9.

[55] Zelis R, Mason DT, Braunwald E. A comparison of the effects of vasodilator stimuli on peripheral resistance vessels in normal subjects and in patients with congestive heart failure. The Journal of clinical investigation. 1968;47:960-70.

[56] Zelis R, Flaim SF. Alterations in vasomotor tone in congestive heart failure. Progress in cardiovascular diseases. 1982;24:437-59.

[57] Link RE, Desai K, Hein L, Stevens ME, Chruscinski A, Bernstein D, et al. Cardiovascular regulation in mice lacking alpha2-adrenergic receptor subtypes b and c. Science. 1996;273:803-5.

[58] Okonko DO, Anker SD. Anemia in chronic heart failure: pathogenetic mechanisms. J Card Fail. 2004;10:S5-9.

[59] Khalid L, Dhakam SH. A review of cardiogenic shock in acute myocardial infarction. Current cardiology reviews. 2008;4:34-40.

[60] DiBona GF, Herman PJ, Sawin LL. Neural control of renal function in edema-forming states. The American journal of physiology. 1988;254:R1017-24.

[61] Sica DA. Sodium and water retention in heart failure and diuretic therapy: basic mechanisms. Cleveland Clinic journal of medicine. 2006;73 Suppl 2:S2-7; discussion S30-3.

[62] Hasking GJ, Esler MD, Jennings GL, Burton D, Johns JA, Korner PI. Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity. Circulation. 1986;73:615-21.

[63] Shearer F, Lang CC, Struthers AD. Renin-angiotensin-aldosterone system inhibitors in heart failure. Clin Pharmacol Ther. 2013;94:459-67.

[64] Hackenthal E, Paul M, Ganten D, Taugner R. Morphology, physiology, and molecular biology of renin secretion. Physiol Rev. 1990;70:1067-116.

[65] Persson PB, Skalweit A, Thiele BJ. Controlling the release and production of renin. Acta Physiol Scand. 2004;181:375-81.

[66] Deschepper CF. Angiotensinogen: hormonal regulation and relative importance in the generation of angiotensin II. Kidney international. 1994;46:1561-3.

[67] Hall JE. Historical perspective of the renin-angiotensin system. Molecular biotechnology. 2003;24:27-39.

[68] Corvol P, Michaud A, Soubrier F, Williams TA. Recent advances in knowledge of the structure and function of the angiotensin I converting enzyme. Journal of hypertension Supplement : official journal of the International Society of Hypertension. 1995;13:S3-10.

[69] Soubrier F, Hubert C, Testut P, Nadaud S, Alhenc-Gelas F, Corvol P. Molecular biology of the angiotensin I converting enzyme: I. Biochemistry and structure of the gene. Journal of hypertension. 1993;11:471-6.

[70] Wei L, Alhenc-Gelas F, Soubrier F, Michaud A, Corvol P, Clauser E. Expression and characterization of recombinant human angiotensin I-converting enzyme. Evidence for a C-terminal transmembrane anchor and for a proteolytic processing of the secreted recombinant and plasma enzymes. The Journal of biological chemistry. 1991;266:5540-6.

[71] Kuoppala A, Lindstedt KA, Saarinen J, Kovanen PT, Kokkonen JO. Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma. American journal of physiology Heart and circulatory physiology. 2000;278:H1069-74.

[72] Dendorfer A, Wolfrum S, Wagemann M, Qadri F, Dominiak P. Pathways of bradykinin degradation in blood and plasma of normotensive and hypertensive rats. American journal of physiology Heart and circulatory physiology. 2001;280:H2182-8.

[73] Rocha ESM, Beraldo WT, Rosenfeld G. Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. The American journal of physiology. 1949;156:261-73.

[74] Kuga T, Mohri M, Egashira K, Hirakawa Y, Tagawa T, Shimokawa H, et al. Bradykinin-induced vasodilation of human coronary arteries in vivo: role of nitric oxide and angiotensin-converting enzyme. J Am Coll Cardiol. 1997;30:108-12.

[75] Needleman P, Key SL, Denny SE, Isakson PC, Marshall GR. Mechanism and modification of bradykinin-induced coronary vasodilation. Proceedings of the National Academy of Sciences of the United States of America. 1975;72:2060-3.

[76] Lamontagne D, Konig A, Bassenge E, Busse R. Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. Journal of cardiovascular pharmacology. 1992;20:652-7.

[77] Iwai N, Inagami T. Identification of two subtypes in the rat type I angiotensin II receptor. FEBS letters. 1992;298:257-60.

[78] de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacological reviews. 2000;52:415-72.

[79] Nouet S, Nahmias C. Signal transduction from the angiotensin II AT2 receptor. Trends in endocrinology and metabolism: TEM. 2000;11:1-6.

[80] Stoll M, Steckelings UM, Paul M, Bottari SP, Metzger R, Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. The Journal of clinical investigation. 1995;95:651-7.

[81] Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, et al. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. The Journal of biological chemistry. 1995;270:18719-22.

[82] Oliverio MI, Best CF, Kim HS, Arendshorst WJ, Smithies O, Coffman TM. Angiotensin II responses in AT1A receptor-deficient mice: a role for AT1B receptors in blood pressure regulation. The American journal of physiology. 1997;272:F515-20.

[83] Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, et al. Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. Proceedings of the National Academy of Sciences of the United States of America. 2006;103:16284-9.

[84] Sadoshima J, Izumo S. Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. Circulation research. 1993;73:413-23.

[85] Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell. 1993;75:977-84.

[86] Shanmugam P, Valente AJ, Prabhu SD, Venkatesan B, Yoshida T, Delafontaine P, et al. Angiotensin-II type 1 receptor and NOX2 mediate TCF/LEF and CREB dependent WISP1 induction and cardiomyocyte hypertrophy. Journal of molecular and cellular cardiology. 2011;50:928-38.

[87] Zhang ZH, Francis J, Weiss RM, Felder RB. The renin-angiotensin-aldosterone system excites hypothalamic paraventricular nucleus neurons in heart failure. American journal of physiology Heart and circulatory physiology. 2002;283:H423-33.

[88] Queiroz-Leite GD, Peruzzetto MC, Neri EA, Reboucas NA. Transcriptional regulation of the Na(+)/H(+) exchanger NHE3 by chronic exposure to angiotensin II in renal epithelial cells. Biochemical and biophysical research communications. 2011;409:470-6.

[89] Kwon TH, Nielsen J, Kim YH, Knepper MA, Frokiaer J, Nielsen S. Regulation of sodium transporters in the thick ascending limb of rat kidney: response to angiotensin II. American journal of physiology Renal physiology. 2003;285:F152-65.

[90] Wolf G, Neilson EG. Angiotensin II induces cellular hypertrophy in cultured murine proximal tubular cells. The American journal of physiology. 1990;259:F768-77.

[91] Wolf G, Mueller E, Stahl RA, Ziyadeh FN. Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-beta. The Journal of clinical investigation. 1993;92:1366-72.

[92] Gurley SB, Riquier-Brison AD, Schnermann J, Sparks MA, Allen AM, Haase VH, et al. AT1A angiotensin receptors in the renal proximal tubule regulate blood pressure. Cell metabolism. 2011;13:469-75.

[93] Hattangady NG, Olala LO, Bollag WB, Rainey WE. Acute and chronic regulation of aldosterone production. Molecular and cellular endocrinology. 2012;350:151-62.

[94] Mizuno Y, Yoshimura M, Yasue H, Sakamoto T, Ogawa H, Kugiyama K, et al. Aldosterone production is activated in failing ventricle in humans. Circulation. 2001;103:72-7.

[95] Quinn SJ, Williams GH. Regulation of aldosterone secretion. Annual review of physiology. 1988;50:409-26.

[96] Ramsay DJ, Keil LC, Sharpe MC, Shinsako J. Angiotensin II infusion increases vasopressin, ACTH, and 11-hydroxycorticosteroid secretion. The American journal of physiology. 1978;234:R66-71.

[97] Hayashi M, Sasaki S, Tsuganezawa H, Monkawa T, Kitajima W, Konishi K, et al. Expression and distribution of aquaporin of collecting duct are regulated by vasopressin V2 receptor in rat kidney. The Journal of clinical investigation. 1994;94:1778-83.

[98] Costello-Boerrigter LC, Smith WB, Boerrigter G, Ouyang J, Zimmer CA, Orlandi C, et al. Vasopressin-2-receptor antagonism augments water excretion without changes in renal hemodynamics or sodium and potassium excretion in human heart failure. American journal of physiology Renal physiology. 2006;290:F273-8.

[99] Steppan J, Nyhan SM, Sikka G, Uribe J, Ahuja A, White AR, et al. Vasopressin-mediated enhancement of adrenergic vasoconstriction involves both the tyrosine kinase and the protein kinase C pathways. Anesthesia and analgesia. 2012;115:1290-5.

[100] Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. Handbook of experimental pharmacology. 2006:213-54.

[101] Forstermann U, Sessa WC. Nitric oxide synthases: regulation and function. European heart journal. 2012;33:829-37, 37a-37d.

[102] Mayer B, Hemmens B. Biosynthesis and action of nitric oxide in mammalian cells. Trends in biochemical sciences. 1997;22:477-81.

[103] Schena M, Mulatero P, Schiavone D, Mengozzi G, Tesio L, Chiandussi L, et al. Vasoactive hormones induce nitric oxide synthase mRNA expression and nitric oxide production in human endothelial cells and monocytes. American journal of hypertension. 1999;12:388-97.

[104] Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. The Biochemical journal. 2001;357:593-615.

[105] Martinez-Ruiz A, Cadenas S, Lamas S. Nitric oxide signaling: classical, less classical, and nonclassical mechanisms. Free radical biology & medicine. 2011;51:17-29.

[106] Ignarro LJ. Haem-dependent activation of cytosolic guanylate cyclase by nitric oxide: a widespread signal transduction mechanism. Biochemical Society transactions. 1992;20:465-9.

[107] Bogdan C. Nitric oxide and the immune response. Nature immunology. 2001;2:907-16.

[108] Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. British journal of pharmacology. 1992;105:575-80.

[109] Heusch P, Aker S, Boengler K, Deindl E, van de Sand A, Klein K, et al. Increased inducible nitric oxide synthase and arginase II expression in heart failure: no net nitrite/nitrate production and protein S-nitrosylation. American journal of physiology Heart and circulatory physiology. 2010;299:H446-53.

[110] Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, et al. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. The Journal of clinical investigation. 1996;97:1916-23.

[111] Ushio-Fukai M, Zafari AM, Fukui T, Ishizaka N, Griendling KK. p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. The Journal of biological chemistry. 1996;271:23317-21.

[112] Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG, et al. Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy. Hypertension. 1998;32:488-95.

[113] Vejlstrup NG, Bouloumie A, Boesgaard S, Andersen CB, Nielsen-Kudsk JE, Mortensen SA, et al. Inducible nitric oxide synthase (iNOS) in the human heart: expression and localization in congestive heart failure. Journal of molecular and cellular cardiology. 1998;30:1215-23.

[114] Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F. Induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. Hypertension. 1992;19:753-7.

[115] Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, et al. Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. Hypertension. 1991;18:165-70.

[116] Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332:411-5.

[117] Merkus D, Brzezinska AK, Zhang C, Saito S, Chilian WM. Cardiac myocytes control release of endothelin-1 in coronary vasculature. American journal of physiology Heart and circulatory physiology. 2005;288:H2088-92.

[118] Hishikawa K, Nakaki T, Marumo T, Suzuki H, Kato R, Saruta T. Pressure enhances endothelin-1 release from cultured human endothelial cells. Hypertension. 1995;25:449-52.

[119] Hasdai D, Holmes DR, Jr., Garratt KN, Edwards WD, Lerman A. Mechanical pressure and stretch release endothelin-1 from human atherosclerotic coronary arteries in vivo. Circulation. 1997;95:357-62.

[120] Kuchan MJ, Frangos JA. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. The American journal of physiology. 1993;264:H150-6.

[121] Cannan CR, Burnett JC, Jr., Brandt RR, Lerman A. Endothelin at pathophysiological concentrations mediates coronary vasoconstriction via the endothelin-A receptor. Circulation. 1995;92:3312-7.

[122] Douglas SA, Ohlstein EH. Signal transduction mechanisms mediating the vascular actions of endothelin. Journal of vascular research. 1997;34:152-64.

[123] Yorikane R, Sakai S, Miyauchi T, Sakurai T, Sugishita Y, Goto K. Increased production of endothelin-1 in the hypertrophied rat heart due to pressure overload. FEBS letters. 1993;332:31-4.

[124] Liang L, Yu J, Zhou W, Liu N, E LL, Wang DS, et al. Endothelin-1 stimulates proinflammatory cytokine expression in human periodontal ligament cells via mitogen-activated protein kinase pathway. Journal of periodontology. 2014;85:618-26.

[125] Molero L, Farre J, Garcia-Mendez A, Jimenez Mateos-Caceres P, Carrasco Martin C, Millas I, et al. Endothelin-1 induced proinflammatory markers in the myocardium and leukocytes of guinea-pigs: role of glycoprotein IIB/IIIA receptors. Cardiovasc Res. 2003;57:109-18.

[126] Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulation. 1999;100:999-1008.

[127] Braunwald E, Bristow MR. Congestive heart failure: fifty years of progress. Circulation. 2000;102:IV14-23.

[128] Floras JS. Sympathetic nervous system activation in human heart failure: clinical implications of an updated model. J Am Coll Cardiol. 2009;54:375-85.

[129] Massie BM. Is neurohormonal activation deleterious to the long-term outcome of patients with congestive heart failure? I. Introduction. J Am Coll Cardiol. 1988;12:547-50.

[130] Grassi G, Seravalle G, Bertinieri G, Turri C, Stella ML, Scopelliti F, et al. Sympathetic and reflex abnormalities in heart failure secondary to ischaemic or idiopathic dilated cardiomyopathy. Clin Sci (Lond). 2001;101:141-6.

[131] Grassi G, Seravalle G, Cattaneo BM, Lanfranchi A, Vailati S, Giannattasio C, et al. Sympathetic activation and loss of reflex sympathetic control in mild congestive heart failure. Circulation. 1995;92:3206-11.

[132] Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature. 2002;415:206-12.

[133] DiPiro JT, Talbert, Robert L., Yee, Gary C., Matzke, Gary R., Wells, Barbara G., Posey, L. Michael. Pharmacotherapy: A Pathophysiological Approach. 7th ed. New York, NY: McGraw-Hill; 2008.

[134] Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G, Butler J. The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. J Am Coll Cardiol. 2009;54:1747-62.

[135] Grassi G, Seravalle G, Quarti-Trevano F, Dell'Oro R, Arenare F, Spaziani D, et al. Sympathetic and baroreflex cardiovascular control in hypertension-related left ventricular dysfunction. Hypertension. 2009;53:205-9.

[136] Zucker IH, Hackley JF, Cornish KG, Hiser BA, Anderson NR, Kieval R, et al. Chronic baroreceptor activation enhances survival in dogs with pacing-induced heart failure. Hypertension. 2007;50:904-10.

[137] Bristow MR. Mechanism of action of beta-blocking agents in heart failure. The American journal of cardiology. 1997;80:26L-40L.

[138] Chakraborti S, Chakraborti T, Shaw G. beta-adrenergic mechanisms in cardiac diseases: a perspective. Cellular signalling. 2000;12:499-513.

[139] Mann DL, Kent RL, Parsons B, Cooper Gt. Adrenergic effects on the biology of the adult mammalian cardiocyte. Circulation. 1992;85:790-804.

[140] Timmermans PB, Chiu AT, Thoolen MJ. Calcium handling in vasoconstriction to stimulation of alpha 1- and alpha 2-adrenoceptors. Canadian journal of physiology and pharmacology. 1987;65:1649-57.

[141] Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. J Am Coll Cardiol. 2013;62:e147-239.

[142] Hjalmarson A, Goldstein S, Fagerberg B, Wedel H, Waagstein F, Kjekshus J, et al. Effect of metoprolol CR XL in chronic heart failure: Metoprolol CR XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF). Lancet. 1999;353:2001-7.

[143] Poole-Wilson PA, Swedberg K, Cleland JGF, Di Lenarda A, Hanrath P, Komajda M, et al. Comparison of carvedilol and metoprolol on clinical outcomes in patients with chronic heart failure in the Carvedilol Or Metoprolol, European Trial (COMET): randomised controlled trial. Lancet. 2003;362:7-13.

[144] Wollert KC, Drexler H. Carvedilol Prospective Randomized Cumulative Survival (COPERNICUS) trial - Carvedilol as the sun and center of the beta-blocker world? Circulation. 2002;106:2164-6.

[145] Iwata M, Yoshikawa T, Baba A, Anzai T, Mitamura H, Ogawa S. Autoantibodies against the second extracellular loop of beta1-adrenergic receptors predict ventricular tachycardia and sudden death in patients with idiopathic dilated cardiomyopathy. J Am Coll Cardiol. 2001;37:418-24.

[146] Peukert S, Fu MLX, Eftekhari P, Poepping I, Voss A, Thalhammer C, et al. The frequency of occurrence of anti-cardiac receptor autoantibodies and their correlation with clinical manifestation in patients with hypertrophic cardiomyopathy. Autoimmunity. 1999;29:291-7.

[147] Magnusson Y, Wallukat G, Waagstein F, Hjalmarson A, Hoebeke J. Autoimmunity in Idiopathic Dilated Cardiomyopathy - Characterization of Antibodies against the Beta(1)-Adrenoceptor with Positive Chronotropic Effect. Circulation. 1994;89:2760-7.

[148] Davies JE, Manisty CH, Petraco R, Barron AJ, Unsworth B, Mayet J, et al. First-in-man safety evaluation of renal denervation for chronic systolic heart failure: primary outcome from REACH-Pilot study. Int J Cardiol. 2013;162:189-92.

[149] Brandt MC, Mahfoud F, Reda S, Schirmer SH, Erdmann E, Bohm M, et al. Renal sympathetic denervation reduces left ventricular hypertrophy and improves cardiac function in patients with resistant hypertension. J Am Coll Cardiol. 2012;59:901-9.

[150] Linz D, Mahfoud F, Schotten U, Ukena C, Neuberger HR, Wirth K, et al. Renal sympathetic denervation suppresses postapneic blood pressure rises and atrial fibrillation in a model for sleep apnea. Hypertension. 2012;60:172-8.

[151] Ukena C, Mahfoud F, Spies A, Kindermann I, Linz D, Cremers B, et al. Effects of renal sympathetic denervation on heart rate and atrioventricular conduction in patients with resistant hypertension. Int J Cardiol. 2013;167:2846-51.

[152] DiFrancesco D, Tortora P. Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. Nature. 1991;351:145-7.

[153] Kimura H, Oyamada Y, Ohshika H, Mori M, Oyamada M. Reversible inhibition of gap junctional intercellular communication, synchronous contraction, and synchronism of

intracellular Ca2+ fluctuation in cultured neonatal rat cardiac myocytes by heptanol. Experimental cell research. 1995;220:348-56.

[154] Bers DM. Cardiac excitation-contraction coupling. Nature. 2002;415:198-205.

[155] Heinzel FR, Bito V, Volders PG, Antoons G, Mubagwa K, Sipido KR. Spatial and temporal inhomogeneities during Ca2+ release from the sarcoplasmic reticulum in pig ventricular myocytes. Circulation research. 2002;91:1023-30.

[156] Soeller C, Cannell MB. Examination of the transverse tubular system in living cardiac rat myocytes by 2-photon microscopy and digital image-processing techniques. Circulation research. 1999;84:266-75.

[157] He J, Conklin MW, Foell JD, Wolff MR, Haworth RA, Coronado R, et al. Reduction in density of transverse tubules and L-type Ca(2+) channels in canine tachycardia-induced heart failure. Cardiovasc Res. 2001;49:298-307.

[158] Chen X, Piacentino V, 3rd, Furukawa S, Goldman B, Margulies KB, Houser SR. L-type Ca2+ channel density and regulation are altered in failing human ventricular myocytes and recover after support with mechanical assist devices. Circulation research. 2002;91:517-24.

[159] Schroder F, Handrock R, Beuckelmann DJ, Hirt S, Hullin R, Priebe L, et al. Increased availability and open probability of single L-type calcium channels from failing compared with nonfailing human ventricle. Circulation. 1998;98:969-76.

[160] Kawai M, Hussain M, Orchard CH. Excitation-contraction coupling in rat ventricular myocytes after formamide-induced detubulation. The American journal of physiology. 1999;277:H603-9.

[161] Scriven DR, Dan P, Moore ED. Distribution of proteins implicated in excitation-contraction coupling in rat ventricular myocytes. Biophysical journal. 2000;79:2682-91.

[162] Jurevicius J, Fischmeister R. cAMP compartmentation is responsible for a local activation of cardiac Ca2+ channels by beta-adrenergic agonists. Proceedings of the National Academy of Sciences of the United States of America. 1996;93:295-9.

[163] Laflamme MA, Becker PL. G(s) and adenylyl cyclase in transverse tubules of heart: implications for cAMP-dependent signaling. The American journal of physiology. 1999;277:H1841-8.

[164] Katz AM, Repke DI. Quantitative aspects of dog cardiac microsomal calcium binding and calcium uptake. Circulation research. 1967;21:153-62.

[165] MacLennan DH. Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. The Journal of biological chemistry. 1970;245:4508-18.

[166] Inui M, Saito A, Fleischer S. Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. The Journal of biological chemistry. 1987;262:15637-42.

[167] Brotto MA, Creazzo TL. Ca2+ transients in embryonic chick heart: contributions from Ca2+ channels and the sarcoplasmic reticulum. The American journal of physiology. 1996;270:H518-25.

[168] Nakanishi T, Okuda H, Kamata K, Abe K, Sekiguchi M, Takao A. Development of myocardial contractile system in the fetal rabbit. Pediatric research. 1987;22:201-7.

[169] Balshaw DM, Xu L, Yamaguchi N, Pasek DA, Meissner G. Calmodulin binding and inhibition of cardiac muscle calcium release channel (ryanodine receptor). The Journal of biological chemistry. 2001;276:20144-53.

[170] Reiken S, Gaburjakova M, Guatimosim S, Gomez AM, D'Armiento J, Burkhoff D, et al. Protein kinase A phosphorylation of the cardiac calcium release channel (ryanodine receptor) in normal and failing hearts. Role of phosphatases and response to isoproterenol. The Journal of biological chemistry. 2003;278:444-53.

[171] Ellison GM, Torella D, Karakikes I, Purushothaman S, Curcio A, Gasparri C, et al. Acute beta-adrenergic overload produces myocyte damage through calcium leakage from the ryanodine receptor 2 but spares cardiac stem cells. The Journal of biological chemistry. 2007;282:11397-409.

[172] Marx SO, Reiken S, Hisamatsu Y, Jayaraman T, Burkhoff D, Rosemblit N, et al. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. Cell. 2000;101:365-76.

[173] Moschella MC, Marks AR. Inositol 1,4,5-trisphosphate receptor expression in cardiac myocytes. The Journal of cell biology. 1993;120:1137-46.

[174] Perez PJ, Ramos-Franco J, Fill M, Mignery GA. Identification and functional reconstitution of the type 2 inositol 1,4,5-trisphosphate receptor from ventricular cardiac myocytes. The Journal of biological chemistry. 1997;272:23961-9.

[175] Domeier TL, Zima AV, Maxwell JT, Huke S, Mignery GA, Blatter LA. IP3 receptor-dependent Ca2+ release modulates excitation-contraction coupling in rabbit ventricular myocytes. American journal of physiology Heart and circulatory physiology. 2008;294:H596-604.

[176] Lipp P, Laine M, Tovey SC, Burrell KM, Berridge MJ, Li W, et al. Functional InsP3 receptors that may modulate excitation-contraction coupling in the heart. Current biology : CB. 2000;10:939-42.

[177] Nakayama H, Bodi I, Maillet M, DeSantiago J, Domeier TL, Mikoshiba K, et al. The IP3 receptor regulates cardiac hypertrophy in response to select stimuli. Circulation research. 2010;107:659-66.

[178] Kockskamper J, Seidlmayer L, Walther S, Hellenkamp K, Maier LS, Pieske B. Endothelin-1 enhances nuclear Ca2+ transients in atrial myocytes through Ins(1,4,5)P3-dependent Ca2+ release from perinuclear Ca2+ stores. Journal of cell science. 2008;121:186-95.

[179] Higazi DR, Fearnley CJ, Drawnel FM, Talasila A, Corps EM, Ritter O, et al. Endothelin-1-stimulated InsP3-induced Ca2+ release is a nexus for hypertrophic signaling in cardiac myocytes. Molecular cell. 2009;33:472-82.

[180] Harzheim D, Movassagh M, Foo RS, Ritter O, Tashfeen A, Conway SJ, et al. Increased InsP3Rs in the junctional sarcoplasmic reticulum augment Ca2+ transients and arrhythmias associated with cardiac hypertrophy. Proceedings of the National Academy of Sciences of the United States of America. 2009;106:11406-11.

[181] Herzog W, Duvall M, Leonard TR. Molecular mechanisms of muscle force regulation: a role for titin? Exercise and sport sciences reviews. 2012;40:50-7.

[182] Huxley HE. The contractile structure of cardiac and skeletal muscle. Circulation. 1961;24:328-35.

[183] Fabiato A, Fabiato F. Dependence of the contractile activation of skinned cardiac cells on the sarcomere length. Nature. 1975;256:54-6.

[184] Weber A, Winicur S. The role of calcium in the superprecipitation of actomyosin. The Journal of biological chemistry. 1961;236:3198-202.

[185] Ebashi S, Lipmann F. Adenosine Triphosphate-Linked Concentration of Calcium Ions in a Particulate Fraction of Rabbit Muscle. The Journal of cell biology. 1962;14:389-400.

[186] Katz AM. Purification and properties of a tropomyosin-containing protein fraction that sensitizes reconstituted actomyosin to calcium-binding agents. The Journal of biological chemistry. 1966;241:1522-9.

[187] Ebashi S, Kodama A. A new protein factor promoting aggregation of tropomyosin. Journal of biochemistry. 1965;58:107-8.

[188] Greaser ML, Gergely J. Reconstitution of troponin activity from three protein components. The Journal of biological chemistry. 1971;246:4226-33.

[189] Zot HG, Potter JD. A structural role for the Ca2+-Mg2+ sites on troponin C in the regulation of muscle contraction. Preparation and properties of troponin C depleted myofibrils. The Journal of biological chemistry. 1982;257:7678-83.

[190] Farah CS, Reinach FC. The troponin complex and regulation of muscle contraction. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1995;9:755-67.

[191] Van Eyk JE, Thomas LT, Tripet B, Wiesner RJ, Pearlstone JR, Farah CS, et al. Distinct regions of troponin I regulate Ca2+-dependent activation and Ca2+ sensitivity of the acto-S1-TM ATPase activity of the thin filament. The Journal of biological chemistry. 1997;272:10529-37.

[192] Landesberg A, Sideman S. Coupling calcium binding to troponin C and cross-bridge cycling in skinned cardiac cells. The American journal of physiology. 1994;266:H1260-71.

[193] Holmes KC. The swinging lever-arm hypothesis of muscle contraction. Current biology : CB. 1997;7:R112-8.

[194] Goldman YE. Kinetics of the actomyosin ATPase in muscle fibers. Annual review of physiology. 1987;49:637-54.

[195] Goldman YE, Hibberd MG, Trentham DR. Relaxation of rabbit psoas muscle fibres from rigor by photochemical generation of adenosine-5'-triphosphate. The Journal of physiology. 1984;354:577-604.

[196] Brini M, Carafoli E. The plasma membrane Ca(2)+ ATPase and the plasma membrane sodium calcium exchanger cooperate in the regulation of cell calcium. Cold Spring Harbor perspectives in biology. 2011;3.

[197] Bers DM, Bassani JW, Bassani RA. Competition and redistribution among calcium transport systems in rabbit cardiac myocytes. Cardiovasc Res. 1993;27:1772-7.

[198] Hao L, Rigaud JL, Inesi G. Ca2+/H+ countertransport and electrogenicity in proteoliposomes containing erythrocyte plasma membrane Ca-ATPase and exogenous lipids. The Journal of biological chemistry. 1994;269:14268-75.

[199] Niggli V, Sigel E, Carafoli E. The purified Ca2+ pump of human erythrocyte membranes catalyzes an electroneutral Ca2+-H+ exchange in reconstituted liposomal systems. The Journal of biological chemistry. 1982;257:2350-6.

[200] Hammes A, Oberdorf-Maass S, Rother T, Nething K, Gollnick F, Linz KW, et al. Overexpression of the sarcolemmal calcium pump in the myocardium of transgenic rats. Circulation research. 1998;83:877-88.

[201] Bridge JH, Smolley JR, Spitzer KW. The relationship between charge movements associated with ICa and INa-Ca in cardiac myocytes. Science. 1990;248:376-8.

[202] Nicoll DA, Longoni S, Philipson KD. Molecular cloning and functional expression of the cardiac sarcolemmal Na(+)-Ca2+ exchanger. Science. 1990;250:562-5.

[203] Pott C, Henderson SA, Goldhaber JI, Philipson KD. Na+/Ca2+ exchanger knockout mice: plasticity of cardiac excitation-contraction coupling. Annals of the New York Academy of Sciences. 2007;1099:270-5.

[204] Iwamoto T, Pan Y, Wakabayashi S, Imagawa T, Yamanaka HI, Shigekawa M. Phosphorylation-dependent regulation of cardiac Na+/Ca2+ exchanger via protein kinase C. The Journal of biological chemistry. 1996;271:13609-15.

[205] Ruknudin A, He S, Lederer WJ, Schulze DH. Functional differences between cardiac and renal isoforms of the rat Na+-Ca2+ exchanger NCX1 expressed in Xenopus oocytes. The Journal of physiology. 2000;529 Pt 3:599-610.

[206] Pogwizd SM, Schlotthauer K, Li L, Yuan W, Bers DM. Arrhythmogenesis and contractile dysfunction in heart failure: Roles of sodium-calcium exchange, inward rectifier potassium current, and residual beta-adrenergic responsiveness. Circulation research. 2001;88:1159-67.

[207] Nagy ZA, Virag L, Toth A, Biliczki P, Acsai K, Banyasz T, et al. Selective inhibition of sodium-calcium exchanger by SEA-0400 decreases early and delayed after depolarization in canine heart. British journal of pharmacology. 2004;143:827-31.

[208] Despa S, Islam MA, Weber CR, Pogwizd SM, Bers DM. Intracellular Na(+) concentration is elevated in heart failure but Na/K pump function is unchanged. Circulation. 2002;105:2543-8.

[209] Sipido KR, Volders PG, de Groot SH, Verdonck F, Van de Werf F, Wellens HJ, et al. Enhanced Ca(2+) release and Na/Ca exchange activity in hypertrophied canine ventricular myocytes: potential link between contractile adaptation and arrhythmogenesis. Circulation. 2000;102:2137-44.

[210] Negretti N, O'Neill SC, Eisner DA. The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. Cardiovasc Res. 1993;27:1826-30.

[211] Yano K, Zarain-Herzberg A. Sarcoplasmic reticulum calsequestrins: structural and functional properties. Mol Cell Biochem. 1994;135:61-70.

[212] Terentyev D, Viatchenko-Karpinski S, Gyorke I, Volpe P, Williams SC, Gyorke S. Calsequestrin determines the functional size and stability of cardiac intracellular calcium stores: Mechanism for hereditary arrhythmia. Proceedings of the National Academy of Sciences of the United States of America. 2003;100:11759-64.

[213] Viatchenko-Karpinski S, Terentyev D, Gyorke I, Terentyeva R, Volpe P, Priori SG, et al. Abnormal calcium signaling and sudden cardiac death associated with mutation of calsequestrin. Circulation research. 2004;94:471-7.

[214] Mercadier JJ, Lompre AM, Duc P, Boheler KR, Fraysse JB, Wisnewsky C, et al. Altered sarcoplasmic reticulum Ca2(+)-ATPase gene expression in the human ventricle during end-stage heart failure. The Journal of clinical investigation. 1990;85:305-9.

[215] Arai M, Alpert NR, MacLennan DH, Barton P, Periasamy M. Alterations in sarcoplasmic reticulum gene expression in human heart failure. A possible mechanism

for alterations in systolic and diastolic properties of the failing myocardium. Circulation research. 1993;72:463-9.

[216] Arnaudeau S, Kelley WL, Walsh JV, Jr., Demaurex N. Mitochondria recycle Ca(2+) to the endoplasmic reticulum and prevent the depletion of neighboring endoplasmic reticulum regions. The Journal of biological chemistry. 2001;276:29430-9.

[217] Chalmers S, Nicholls DG. The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria. The Journal of biological chemistry. 2003;278:19062-70.

[218] Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, et al. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature. 2011;476:341-5.

[219] De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature. 2011;476:336-40.

[220] Csordas G, Golenar T, Seifert EL, Kamer KJ, Sancak Y, Perocchi F, et al. MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca(2)(+) uniporter. Cell metabolism. 2013;17:976-87.

[221] McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol Rev. 1990;70:391-425.

[222] Brandes R, Bers DM. Simultaneous measurements of mitochondrial NADH and Ca(2+) during increased work in intact rat heart trabeculae. Biophysical journal. 2002;83:587-604.

[223] Moravec CS, Bond M. Effect of inotropic stimulation on mitochondrial calcium in cardiac muscle. The Journal of biological chemistry. 1992;267:5310-6.

[224] Gunter TE, Buntinas L, Sparagna G, Eliseev R, Gunter K. Mitochondrial calcium transport: mechanisms and functions. Cell calcium. 2000;28:285-96.

[225] Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. Biochemical Society transactions. 2006;34:232-7.

[226] Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res. 2004;61:372-85.

[227] Crompton M, Virji S, Doyle V, Johnson N, Ward JM. The mitochondrial permeability transition pore. Biochemical Society symposium. 1999;66:167-79.

[228] Griffiths EJ, Halestrap AP. Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. Journal of molecular and cellular cardiology. 1993;25:1461-9.

[229] Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. Circulation research. 2000;86:152-7.

[230] Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. The Journal of experimental medicine. 2000;192:1001-14.

[231] Bittl JA, Ingwall JS. Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart. A 31P NMR magnetization transfer study. The Journal of biological chemistry. 1985;260:3512-7.

[232] Goodwin GW, Arteaga JR, Taegtmeyer H. Glycogen turnover in the isolated working rat heart. The Journal of biological chemistry. 1995;270:9234-40.

[233] Wallimann T, Dolder M, Schlattner U, Eder M, Hornemann T, O'Gorman E, et al. Some new aspects of creatine kinase (CK): compartmentation, structure, function and regulation for cellular and mitochondrial bioenergetics and physiology. BioFactors. 1998;8:229-34.

[234] Boehm E, Chan S, Monfared M, Wallimann T, Clarke K, Neubauer S. Creatine transporter activity and content in the rat heart supplemented by and depleted of creatine. American journal of physiology Endocrinology and metabolism. 2003;284:E399-406.

[235] Neubauer S, Remkes H, Spindler M, Horn M, Wiesmann F, Prestle J, et al. Downregulation of the Na(+)-creatine cotransporter in failing human myocardium and in experimental heart failure. Circulation. 1999;100:1847-50.

[236] Ten Hove M, Chan S, Lygate C, Monfared M, Boehm E, Hulbert K, et al. Mechanisms of creatine depletion in chronically failing rat heart. Journal of molecular and cellular cardiology. 2005;38:309-13.

[237] Nakae I, Mitsunami K, Omura T, Yabe T, Tsutamoto T, Matsuo S, et al. Proton magnetic resonance spectroscopy can detect creatine depletion associated with the progression of heart failure in cardiomyopathy. J Am Coll Cardiol. 2003;42:1587-93.

[238] Shen W, Asai K, Uechi M, Mathier MA, Shannon RP, Vatner SF, et al. Progressive loss of myocardial ATP due to a loss of total purines during the development of heart failure in dogs: a compensatory role for the parallel loss of creatine. Circulation. 1999;100:2113-8.

[239] Neubauer S, Horn M, Cramer M, Harre K, Newell JB, Peters W, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. Circulation. 1997;96:2190-6.

[240] Hansch A, Rzanny R, Heyne JP, Leder U, Reichenbach JR, Kaiser WA. Noninvasive measurements of cardiac high-energy phosphate metabolites in dilated cardiomyopathy by using 31P spectroscopic chemical shift imaging. Eur Radiol. 2005;15:319-23.

[241] Beer M, Seyfarth T, Sandstede J, Landschutz W, Lipke C, Kostler H, et al. Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with (31)P-SLOOP magnetic resonance spectroscopy. J Am Coll Cardiol. 2002;40:1267-74.

[242] Starling RC, Hammer DF, Altschuld RA. Human myocardial ATP content and in vivo contractile function. Mol Cell Biochem. 1998;180:171-7.

[243] Nascimben L, Ingwall JS, Pauletto P, Friedrich J, Gwathmey JK, Saks V, et al. Creatine kinase system in failing and nonfailing human myocardium. Circulation. 1996;94:1894-901.

[244] Feygin J, Hu Q, Swingen C, Zhang J. Relationships between regional myocardial wall stress and bioenergetics in hearts with left ventricular hypertrophy. American journal of physiology Heart and circulatory physiology. 2008;294:H2313-21.

[245] Hearse DJ. Oxygen deprivation and early myocardial contractile failure: a reassessment of the possible role of adenosine triphosphate. The American journal of cardiology. 1979;44:1115-21.

[246] Bowers KC, Allshire AP, Cobbold PH. Bioluminescent measurement in single cardiomyocytes of sudden cytosolic ATP depletion coincident with rigor. Journal of molecular and cellular cardiology. 1992;24:213-8.

[247] Tian R, Christe ME, Spindler M, Hopkins JC, Halow JM, Camacho SA, et al. Role of MgADP in the development of diastolic dysfunction in the intact beating rat heart. The Journal of clinical investigation. 1997;99:745-51.

[248] Maack C, O'Rourke B. Excitation-contraction coupling and mitochondrial energetics. Basic Res Cardiol. 2007;102:369-92.

[249] ten Hove M, Lygate CA, Fischer A, Schneider JE, Sang AE, Hulbert K, et al. Reduced inotropic reserve and increased susceptibility to cardiac ischemia/reperfusion injury in phosphocreatine-deficient guanidinoacetate-N-methyltransferase-knockout mice. Circulation. 2005;111:2477-85.

[250] Murray AJ, Cole MA, Lygate CA, Carr CA, Stuckey DJ, Little SE, et al. Increased mitochondrial uncoupling proteins, respiratory uncoupling and decreased efficiency in the chronically infarcted rat heart. Journal of molecular and cellular cardiology. 2008;44:694-700.

[251] Gong G, Liu J, Liang P, Guo T, Hu Q, Ochiai K, et al. Oxidative capacity in failing hearts. American journal of physiology Heart and circulatory physiology. 2003;285:H541-8.

[252] Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R. Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. The Journal of physiology. 2003;551:491-501.

[253] Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. Circulation. 1996;94:2837-42.

[254] Osorio JC, Stanley WC, Linke A, Castellari M, Diep QN, Panchal AR, et al. Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor-alpha in pacing-induced heart failure. Circulation. 2002;106:606-12.

[255] Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. Physiol Rev. 2010;90:207-58.

[256] Wisneski JA, Gertz EW, Neese RA, Mayr M. Myocardial metabolism of free fatty acids. Studies with 14C-labeled substrates in humans. The Journal of clinical investigation. 1987;79:359-66.

[257] Taegtmeyer H, Overturf ML. Effects of moderate hypertension on cardiac function and metabolism in the rabbit. Hypertension. 1988;11:416-26.

[258] Bugger H, Schwarzer M, Chen D, Schrepper A, Amorim PA, Schoepe M, et al. Proteomic remodelling of mitochondrial oxidative pathways in pressure overload-induced heart failure. Cardiovasc Res. 2010;85:376-84.

[259] Morrow DA, Givertz MM. Modulation of myocardial energetics: emerging evidence for a therapeutic target in cardiovascular disease. Circulation. 2005;112:3218-21.

[260] Essop MF, Opie LH. Metabolic therapy for heart failure. European heart journal. 2004;25:1765-8.

[261] Frey N, Brixius K, Schwinger RH, Benis T, Karpowski A, Lorenzen HP, et al. Alterations of tension-dependent ATP utilization in a transgenic rat model of hypertrophic cardiomyopathy. The Journal of biological chemistry. 2006;281:29575-82.

[262] Ashrafian H, Redwood C, Blair E, Watkins H. Hypertrophic cardiomyopathy:a paradigm for myocardial energy depletion. Trends in genetics : TIG. 2003;19:263-8.

[263] Sweeney HL, Feng HS, Yang Z, Watkins H. Functional analyses of troponin T mutations that cause hypertrophic cardiomyopathy: insights into disease pathogenesis and troponin function. Proceedings of the National Academy of Sciences of the United States of America. 1998;95:14406-10.

[264] Sharma S, Adrogue JV, Golfman L, Uray I, Lemm J, Youker K, et al. Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004;18:1692-700.

[265] Marfella R, Di Filippo C, Portoghese M, Barbieri M, Ferraraccio F, Siniscalchi M, et al. Myocardial lipid accumulation in patients with pressure-overloaded heart and metabolic syndrome. Journal of lipid research. 2009;50:2314-23.

[266] Zhou YT, Grayburn P, Karim A, Shimabukuro M, Higa M, Baetens D, et al. Lipotoxic heart disease in obese rats: implications for human obesity. Proceedings of the National Academy of Sciences of the United States of America. 2000;97:1784-9.

[267] Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. The Journal of biological chemistry. 2001;276:14890-5.

[268] Ostrander DB, Sparagna GC, Amoscato AA, McMillin JB, Dowhan W. Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis. The Journal of biological chemistry. 2001;276:38061-7.

[269] Flagg TP, Cazorla O, Remedi MS, Haim TE, Tones MA, Bahinski A, et al. Ca2+-independent alterations in diastolic sarcomere length and relaxation kinetics in a mouse model of lipotoxic diabetic cardiomyopathy. Circulation research. 2009;104:95-103.

[270] Chiu HC, Kovacs A, Blanton RM, Han X, Courtois M, Weinheimer CJ, et al. Transgenic expression of fatty acid transport protein 1 in the heart causes lipotoxic cardiomyopathy. Circulation research. 2005;96:225-33.

[271] Yagyu H, Chen G, Yokoyama M, Hirata K, Augustus A, Kako Y, et al. Lipoprotein lipase (LpL) on the surface of cardiomyocytes increases lipid uptake and produces a cardiomyopathy. The Journal of clinical investigation. 2003;111:419-26.

[272] Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free radical biology & medicine. 2000;28:1456-62.

[273] Hemnani T, Parihar MS. Reactive oxygen species and oxidative DNA damage. Indian journal of physiology and pharmacology. 1998;42:440-52.

[274] Suematsu N, Tsutsui H, Wen J, Kang D, Ikeuchi M, Ide T, et al. Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation. 2003;107:1418-23.

[275] Sabri A, Hughie HH, Lucchesi PA. Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. Antioxidants & redox signaling. 2003;5:731-40.

[276] Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, et al. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II. Circulation. 1998;98:794-9.

[277] Delbosc S, Cristol JP, Descomps B, Mimran A, Jover B. Simvastatin prevents angiotensin II-induced cardiac alteration and oxidative stress. Hypertension. 2002;40:142-7.

[278] Guerra L, Cerbai E, Gessi S, Borea PA, Mugelli A. The effect of oxygen free radicals on calcium current and dihydropyridine binding sites in guinea-pig ventricular myocytes. British journal of pharmacology. 1996;118:1278-84.

[279] Kaplan P, Babusikova E, Lehotsky J, Dobrota D. Free radical-induced protein modification and inhibition of Ca2+-ATPase of cardiac sarcoplasmic reticulum. Mol Cell Biochem. 2003;248:41-7.

[280] He X, Liu Y, Sharma V, Dirksen RT, Waugh R, Sheu SS, et al. ASK1 associates with troponin T and induces troponin T phosphorylation and contractile dysfunction in cardiomyocytes. Am J Pathol. 2003;163:243-51.

[281] Canton M, Neverova I, Menabo R, Van Eyk J, Di Lisa F. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. American journal of physiology Heart and circulatory physiology. 2004;286:H870-7.

[282] Avner BS, Shioura KM, Scruggs SB, Grachoff M, Geenen DL, Helseth DL, Jr., et al. Myocardial infarction in mice alters sarcomeric function via post-translational protein modification. Mol Cell Biochem. 2012;363:203-15.

[283] Eaton P, Byers HL, Leeds N, Ward MA, Shattock MJ. Detection, quantitation, purification, and identification of cardiac proteins S-thiolated during ischemia and reperfusion. The Journal of biological chemistry. 2002;277:9806-11.

[284] Wessler I, Kirkpatrick CJ, Racke K. The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. Clinical and experimental pharmacology & physiology. 1999;26:198-205.

[285] Nachmansohn D, Machado AL. The Formation of Acetylcholine. A New Enzyme: Choline Acetylase. Journal of Neurophysiology. 1943;6:397-403.

[286] Brandon EP, Mellott T, Pizzo DP, Coufal N, D'Amour KA, Gobeske K, et al. Choline transporter 1 maintains cholinergic function in choline acetyltransferase haploinsufficiency. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2004;24:5459-66.

[287] Okuda T, Haga T. High-affinity choline transporter. Neurochemical research. 2003;28:483-8.

[288] Kuhar MJ, Murrin LC. Sodium-dependent, high affinity choline uptake. Journal of neurochemistry. 1978;30:15-21.

[289] Ferguson SM, Bazalakova M, Savchenko V, Tapia JC, Wright J, Blakely RD. Lethal impairment of cholinergic neurotransmission in hemicholinium-3-sensitive choline transporter knockout mice. Proceedings of the National Academy of Sciences of the United States of America. 2004;101:8762-7.

[290] Erickson JD, Varoqui H, Schafer MK, Modi W, Diebler MF, Weihe E, et al. Functional identification of a vesicular acetylcholine transporter and its expression from a "cholinergic" gene locus. The Journal of biological chemistry. 1994;269:21929-32.

[291] Michaelson DM, Angel I. Saturable acetylcholine transport into purified cholinergic synaptic vesicles. Proceedings of the National Academy of Sciences of the United States of America. 1981;78:2048-52.

[292] Toll L, Howard BD. Evidence that an ATPase and a protonmotive force function in the transport of acetylcholine into storage vesicles. The Journal of biological chemistry. 1980;255:1787-9.

[293] Alfonso A, Grundahl K, Duerr JS, Han HP, Rand JB. The Caenorhabditis elegans unc-17 gene: a putative vesicular acetylcholine transporter. Science. 1993;261:617-9.

[294] Roghani A, Feldman J, Kohan SA, Shirzadi A, Gundersen CB, Brecha N, et al. Molecular cloning of a putative vesicular transporter for acetylcholine. Proceedings of the National Academy of Sciences of the United States of America. 1994;91:10620-4.

[295] Bejanin S, Cervini R, Mallet J, Berrard S. A unique gene organization for two cholinergic markers, choline acetyltransferase and a putative vesicular transporter of acetylcholine. The Journal of biological chemistry. 1994;269:21944-7.

[296] Kitamoto T, Wang W, Salvaterra PM. Structure and organization of the Drosophila cholinergic locus. The Journal of biological chemistry. 1998;273:2706-13.

[297] Parsons SM, Prior C, Marshall IG. Acetylcholine transport, storage, and release. International review of neurobiology. 1993;35:279-390.

[298] de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, et al. The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol. 2009;29:5238-50.

[299] Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, et al. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron. 2006;51:601-12.

[300] Song H, Ming G, Fon E, Bellocchio E, Edwards RH, Poo M. Expression of a putative vesicular acetylcholine transporter facilitates quantal transmitter packaging. Neuron. 1997;18:815-26.

[301] Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhaes AC, Feng G, et al. ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;33:10427-38.

[302] Nagy PM, Aubert I. Overexpression of the vesicular acetylcholine transporter increased acetylcholine release in the hippocampus. Neuroscience. 2012;218:1-11.

[303] Brodde OE, Bruck H, Leineweber K, Seyfarth T. Presence, distribution and physiological function of adrenergic and muscarinic receptor subtypes in the human heart. Basic Res Cardiol. 2001;96:528-38.

[304] Agostoni E, Chinnock JE, De Daly MB, Murray JG. Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. The Journal of physiology. 1957;135:182-205.

[305] Sunahara RK, Dessauer CW, Gilman AG. Complexity and diversity of mammalian adenylyl cyclases. Annual review of pharmacology and toxicology. 1996;36:461-80.

[306] Ishikawa Y, Homcy CJ. The adenylyl cyclases as integrators of transmembrane signal transduction. Circulation research. 1997;80:297-304.

[307] Osterrieder W, Brum G, Hescheler J, Trautwein W, Flockerzi V, Hofmann F. Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca2+ current. Nature. 1982;298:576-8.

[308] Logothetis DE, Kurachi Y, Galper J, Neer EJ, Clapham DE. The beta gamma subunits of GTP-binding proteins activate the muscarinic K+ channel in heart. Nature. 1987;325:321-6.

[309] Ponicke K, Heinroth-Hoffmann I, Brodde OE. Demonstration of functional M3-muscarinic receptors in ventricular cardiomyocytes of adult rats. British journal of pharmacology. 2003;138:156-60.

[310] Nishimaru K, Tanaka Y, Tanaka H, Shigenobu K. Positive and negative inotropic effects of muscarinic receptor stimulation in mouse left atria. Life sciences. 2000;66:607-15.

[311] Pan Z, Guo Y, Qi H, Fan K, Wang S, Zhao H, et al. M3 subtype of muscarinic acetylcholine receptor promotes cardioprotection via the suppression of miR-376b-5p. PloS one. 2012;7:e32571.

[312] Liu Y, Wang S, Wang C, Song H, Han H, Hang P, et al. Upregulation of M(3) muscarinic receptor inhibits cardiac hypertrophy induced by angiotensin II. Journal of translational medicine. 2013;11:209.

[313] Habermeier-Muth A, Altes U, Forsyth KM, Muscholl E. A presynaptic excitatory M1 muscarine receptor at postganglionic cardiac noradrenergic nerve fibres that is activated by endogenous acetylcholine. Naunyn-Schmiedeberg's archives of pharmacology. 1990;342:483-9.

[314] Trendelenburg AU, Gomeza J, Klebroff W, Zhou H, Wess J. Heterogeneity of presynaptic muscarinic receptors mediating inhibition of sympathetic transmitter release: a study with M2- and M4-receptor-deficient mice. British journal of pharmacology. 2003;138:469-80.

[315] Liu HR, Zhao RR, Jiao XY, Wang YY, Fu M. Relationship of myocardial remodeling to the genesis of serum autoantibodies to cardiac beta(1)-adrenoceptors and muscarinic type 2 acetylcholine receptors in rats. J Am Coll Cardiol. 2002;39:1866-73.

[316] Hollinger S, Hepler JR. Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacological reviews. 2002;54:527-59.

[317] Heximer SP, Watson N, Linder ME, Blumer KJ, Hepler JR. RGS2/G0S8 is a selective inhibitor of Gqalpha function. Proceedings of the National Academy of Sciences of the United States of America. 1997;94:14389-93.

[318] Chidiac P, Ross EM. Phospholipase C-beta1 directly accelerates GTP hydrolysis by Galphaq and acceleration is inhibited by Gbeta gamma subunits. The Journal of biological chemistry. 1999;274:19639-43.

[319] Heximer SP, Srinivasa SP, Bernstein LS, Bernard JL, Linder ME, Hepler JR, et al. G protein selectivity is a determinant of RGS2 function. The Journal of biological chemistry. 1999;274:34253-9.

[320] Cladman W, Chidiac P. Characterization and comparison of RGS2 and RGS4 as GTPase-activating proteins for m2 muscarinic receptor-stimulated G(i). Molecular pharmacology. 2002;62:654-9.

[321] Roy AA, Nunn C, Ming H, Zou MX, Penninger J, Kirshenbaum LA, et al. Up-regulation of endogenous RGS2 mediates cross-desensitization between Gs and Gq signaling in osteoblasts. The Journal of biological chemistry. 2006;281:32684-93.

[322] Nunn C, Zou MX, Sobiesiak AJ, Roy AA, Kirshenbaum LA, Chidiac P. RGS2 inhibits beta-adrenergic receptor-induced cardiomyocyte hypertrophy. Cellular signalling. 2010;22:1231-9.

[323] Chidiac P, Sobiesiak AJ, Lee KN, Gros R, Nguyen CH. The eIF2B-interacting domain of RGS2 protects against GPCR agonist-induced hypertrophy in neonatal rat cardiomyocytes. Cellular signalling. 2014;26:1226-34.

[324] Heximer SP, Knutsen RH, Sun X, Kaltenbronn KM, Rhee MH, Peng N, et al. Hypertension and prolonged vasoconstrictor signaling in RGS2-deficient mice. The Journal of clinical investigation. 2003;111:445-52.

[325] Takimoto E, Koitabashi N, Hsu S, Ketner EA, Zhang M, Nagayama T, et al. Regulator of G protein signaling 2 mediates cardiac compensation to pressure overload and antihypertrophic effects of PDE5 inhibition in mice. The Journal of clinical investigation. 2009;119:408-20.

[326] Binkley PF, Nunziata E, Haas GJ, Nelson SD, Cody RJ. Parasympathetic withdrawal is an integral component of autonomic imbalance in congestive heart failure: demonstration in human subjects and verification in a paced canine model of ventricular failure. J Am Coll Cardiol. 1991;18:464-72.

[327] Vanoli E, De Ferrari GM, Stramba-Badiale M, Hull SS, Jr., Foreman RD, Schwartz PJ. Vagal stimulation and prevention of sudden death in conscious dogs with a healed myocardial infarction. Circulation research. 1991;68:1471-81.

[328] Billman GE, Schwartz PJ, Stone HL. Baroreceptor reflex control of heart rate: a predictor of sudden cardiac death. Circulation. 1982;66:874-80.

[329] Fu LX, Magnusson Y, Bergh CH, Liljeqvist JA, Waagstein F, Hjalmarson A, et al. Localization of a Functional Autoimmune Epitope on the Muscarinic Acetylcholine Receptor-2 in Patients with Idiopathic Dilated Cardiomyopathy. Journal of Clinical Investigation. 1993;91:1964-8.

[330] Kinugawa T, Dibner-Dunlap ME. Altered vagal and sympathetic control of heart rate in left ventricular dysfunction and heart failure. The American journal of physiology. 1995;268:R310-16.

[331] Bibevski S, Dunlap ME. Ganglionic mechanisms contribute to diminished vagal control in heart failure. Circulation. 1999;99:2958-63.

[332] LaCroix C, Freeling J, Giles A, Wess J, Li YF. Deficiency of M2 muscarinic acetylcholine receptors increases susceptibility of ventricular function to chronic adrenergic stress. American journal of physiology Heart and circulatory physiology. 2008;294:H810-20.

[333] Yan AT, Yan RT, Spinale FG, Afzal R, Gunasinghe HR, Arnold M, et al. Plasma matrix metalloproteinase-9 level is correlated with left ventricular volumes and ejection fraction in patients with heart failure. J Card Fail. 2006;12:514-9.

[334] Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, et al. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol. 2010;30:1746-56.

[335] English BA, Appalsamy M, Diedrich A, Ruggiero AM, Lund D, Wright J, et al. Tachycardia, reduced vagal capacity, and age-dependent ventricular dysfunction arising from diminished expression of the presynaptic choline transporter. Am J Physiol Heart Circ Physiol. 2010;299:H799-810.

[336] Schwartz PJ, De Ferrari GM. Sympathetic-parasympathetic interaction in health and disease: abnormalities and relevance in heart failure. Heart Fail Rev. 2011;16:101-7.

[337] Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyze acetylcholine. Neuroscience. 2002;110:627-39.

[338] Chatonnet A, Lockridge O. Comparison of butyrylcholinesterase and acetylcholinesterase. The Biochemical journal. 1989;260:625-34.

[339] Li B, Stribley JA, Ticu A, Xie W, Schopfer LM, Hammond P, et al. Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. Journal of neurochemistry. 2000;75:1320-31.

[340] Calderon-Margalit R, Adler B, Abramson JH, Gofin J, Kark JD. Butyrylcholinesterase activity, cardiovascular risk factors, and mortality in middle-aged and elderly men and women in Jerusalem. Clinical chemistry. 2006;52:845-52.

[341] Goliasch G, Haschemi A, Marculescu R, Endler G, Maurer G, Wagner O, et al. Butyrylcholinesterase activity predicts long-term survival in patients with coronary artery disease. Clinical chemistry. 2012;58:1055-8.

[342] Goliasch G, Kleber ME, Richter B, Plischke M, Hoke M, Haschemi A, et al. Routinely available biomarkers improve prediction of long-term mortality in stable coronary artery disease: the Vienna and Ludwigshafen Coronary Artery Disease (VILCAD) risk score. European heart journal. 2012;33:2282-9.

[343] Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation. 2004;109:120-4.

[344] Zhang Y, Popovic ZB, Bibevski S, Fakhry I, Sica DA, Van Wagoner DR, et al. Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model. Circ Heart Fail. 2009;2:692-9.

[345] Handa T, Katare RG, Kakinuma Y, Arikawa M, Ando M, Sasaguri S, et al. Anti-Alzheimer's drug, donepezil, markedly improves long-term survival after chronic heart failure in mice. J Card Fail. 2009;15:805-11.

[346] Li M, Zheng C, Kawada T, Inagaki M, Uemura K, Shishido T, et al. Donepezil markedly improves long-term survival in rats with chronic heart failure after extensive myocardial infarction. Circ J. 2013;77:2519-25.

[347] Lataro RM, Silva CA, Fazan R, Jr., Rossi MA, Prado CM, Godinho RO, et al. Increase in parasympathetic tone by pyridostigmine prevents ventricular dysfunction during the onset of heart failure. American journal of physiology Regulatory, integrative and comparative physiology. 2013;305:R908-16.

[348] Schwartz PJ, De Ferrari GM, Sanzo A, Landolina M, Rordorf R, Raineri C, et al. Long term vagal stimulation in patients with advanced heart failure: first experience in man. Eur J Heart Fail. 2008;10:884-91.

[349] De Ferrari GM, Crijns HJ, Borggrefe M, Milasinovic G, Smid J, Zabel M, et al. Chronic vagus nerve stimulation: a new and promising therapeutic approach for chronic heart failure. European heart journal. 2011;32:847-55.

[350] Nordstrom P, Religa D, Wimo A, Winblad B, Eriksdotter M. The use of cholinesterase inhibitors and the risk of myocardial infarction and death: a nationwide cohort study in subjects with Alzheimer's disease. European heart journal. 2013;34:2585-91.

[351] Friedman A, Kaufer D, Shemer J, Hendler I, Soreq H, Tur-Kaspa I. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nature medicine. 1996;2:1382-5.

[352] Dillman JF, 3rd, Phillips CS, Kniffin DM, Tompkins CP, Hamilton TA, Kan RK. Gene expression profiling of rat hippocampus following exposure to the acetylcholinesterase inhibitor soman. Chemical research in toxicology. 2009;22:633-8.

[353] Zhao Y, Wang C, Wu J, Wang Y, Zhu W, Zhang Y, et al. Choline protects against cardiac hypertrophy induced by increased after-load. International journal of biological sciences. 2013;9:295-302.

[354] Wang S, Han HM, Pan ZW, Hang PZ, Sun LH, Jiang YN, et al. Choline inhibits angiotensin II-induced cardiac hypertrophy by intracellular calcium signal and p38 MAPK pathway. Naunyn-Schmiedeberg's archives of pharmacology. 2012;385:823-31.

[355] Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. The Journal of clinical investigation. 2010;120:408-21.

[356] Grando SA, Kist DA, Qi M, Dahl MV. Human keratinocytes synthesize, secrete, and degrade acetylcholine. The Journal of investigative dermatology. 1993;101:32-6.

[357] Reinheimer T, Munch M, Bittinger F, Racke K, Kirkpatrick CJ, Wessler I. Glucocorticoids mediate reduction of epithelial acetylcholine content in the airways of rats and humans. European journal of pharmacology. 1998;349:277-84.

[358] Nguyen VT, Hall LL, Gallacher G, Ndoye A, Jolkovsky DL, Webber RJ, et al. Choline acetyltransferase, acetylcholinesterase, and nicotinic acetylcholine receptors of human gingival and esophageal epithelia. Journal of dental research. 2000;79:939-49.

[359] Maeda S, Jun JG, Kuwahara-Otani S, Tanaka K, Hayakawa T, Seki M. Non-neuronal expression of choline acetyltransferase in the rat kidney. Life sciences. 2011;89:408-14.

[360] Rodriguez-Diaz R, Dando R, Jacques-Silva MC, Fachado A, Molina J, Abdulreda MH, et al. Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans. Nature medicine. 2011;17:888-92.

[361] Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science. 2011;334:98-101.

[362] Fujii T, Tsuchiya T, Yamada S, Fujimoto K, Suzuki T, Kasahara T, et al. Localization and synthesis of acetylcholine in human leukemic T cell lines. J Neurosci Res. 1996;44:66-72.

[363] Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc Res. 2004;62:112-21.

[364] Crick SJ, Wharton J, Sheppard MN, Royston D, Yacoub MH, Anderson RH, et al. Innervation of the human cardiac conduction system. A quantitative immunohistochemical and histochemical study. Circulation. 1994;89:1697-708.

[365] Gatti PJ, Johnson TA, McKenzie J, Lauenstein JM, Gray A, Massari VJ. Vagal control of left ventricular contractility is selectively mediated by a cranioventricular intracardiac ganglion in the cat. Journal of the autonomic nervous system. 1997;66:138-44.

[366] Nakano H, Furukawa Y, Inoue Y, Sawaki S, Oguchi T, Chiba S. Right ventricular responses to vagus stimulation of fibers to discrete cardiac regions in dog hearts. Journal of the autonomic nervous system. 1998;74:179-88.

[367] Lewis ME, Al-Khalidi AH, Bonser RS, Clutton-Brock T, Morton D, Paterson D, et al. Vagus nerve stimulation decreases left ventricular contractility in vivo in the human and pig heart. The Journal of physiology. 2001;534:547-52.

[368] Rysevaite K, Saburkina I, Pauziene N, Vaitkevicius R, Noujaim SF, Jalife J, et al. Immunohistochemical characterization of the intrinsic cardiac neural plexus in whole-mount mouse heart preparations. Heart rhythm : the official journal of the Heart Rhythm Society. 2011;8:731-8.

[369] Zang WJ, Chen LN, Yu XJ. Progress in the study of vagal control of cardiac ventricles. Sheng li xue bao : [Acta physiologica Sinica]. 2005;57:659-72.

[370] Kawano H, Okada R, Yano K. Histological study on the distribution of autonomic nerves in the human heart. Heart and vessels. 2003;18:32-9.

[371] Rana OR, Schauerte P, Kluttig R, Schroder JW, Koenen RR, Weber C, et al. Acetylcholine as an age-dependent non-neuronal source in the heart. Auton Neurosci. 2010;156:82-9.

[372] Kakinuma Y, Akiyama T, Sato T. Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J. 2009;276:5111-25.

[373] Kakinuma Y, Akiyama T, Okazaki K, Arikawa M, Noguchi T, Sato T. A non-neuronal cardiac cholinergic system plays a protective role in myocardium salvage during ischemic insults. PloS one. 2012;7:e50761.

[374] Kakinuma Y, Tsuda M, Okazaki K, Akiyama T, Arikawa M, Noguchi T, et al. Heart-specific overexpression of choline acetyltransferase gene protects murine heart against ischemia through hypoxia-inducible factor-1alpha-related defense mechanisms. Journal of the American Heart Association. 2013;2:e004887.

[375] Tracey KJ. The inflammatory reflex. Nature. 2002;420:853-9.

[376] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature. 2003;421:384-8.

[377] Bellinger DL, Lorton D, Hamill RW, Felten SY, Felten DL. Acetylcholinesterase staining and choline acetyltransferase activity in the young adult rat spleen: lack of evidence for cholinergic innervation. Brain Behav Immun. 1993;7:191-204.

[378] Guarini S, Altavilla D, Cainazzo MM, Giuliani D, Bigiani A, Marini H, et al. Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. Circulation. 2003;107:1189-94.

[379] Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J, et al. Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem. 2004;89:337-43.

[380] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000;405:458-62.

[381] Chen JK, Zhao T, Ni M, Li DJ, Tao X, Shen FM. Downregulation of alpha7 nicotinic acetylcholine receptor in two-kidney one-clip hypertensive rats. BMC cardiovascular disorders. 2012;12:38.

[382] Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. The New England journal of medicine. 1990;323:236-41.

[383] Shan K, Kurrelmeyer K, Seta Y, Wang F, Dibbs Z, Deswal A, et al. The role of cytokines in disease progression in heart failure. Curr Opin Cardiol. 1997;12:218-23.

[384] Mann DL. Inflammatory mediators and the failing heart: past, present, and the foreseeable future. Circulation research. 2002;91:988-98.

[385] Schulte A, Lichtenstern C, Henrich M, Weigand MA, Uhle F. Loss of vagal tone aggravates systemic inflammation and cardiac impairment in endotoxemic rats. The Journal of surgical research. 2014.

[386] Li DJ, Evans RG, Yang ZW, Song SW, Wang P, Ma XJ, et al. Dysfunction of the cholinergic anti-inflammatory pathway mediates organ damage in hypertension. Hypertension. 2011;57:298-307.

[387] Aukrust P, Ueland T, Muller F, Andreassen AK, Nordoy I, Aas H, et al. Elevated circulating levels of C-C chemokines in patients with congestive heart failure. Circulation. 1998;97:1136-43.

[388] Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP, et al. Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. Circ Res. 1997;81:627-35.

[389] Martelli D, Yao ST, McKinley MJ, McAllen RM. Reflex control of inflammation by sympathetic nerves, not the vagus. The Journal of physiology. 2014.

[390] Borovikova LV, Ivanova S, Nardi D, Zhang M, Yang H, Ombrellino M, et al. Role of vagus nerve signaling in CNI-1493-mediated suppression of acute inflammation. Auton Neurosci. 2000;85:141-7.

Chapter 2

AN ANALYSIS OF THE MYOCARDIAL TRANSCRIPTOME IN A MOUSE MODEL OF CARDIAC DYSFUNCTION WITH REDUCED CHOLINERGIC NEUROTRANSMISSION

A version of this chapter has previously been published: Roy A, Lara A, Guimarães D, Pires R, Gomes ER, Carter DE, Gomez MV, Guatimosim S, Prado VF, Prado MA, Gros R. (2012). An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with decreased cholinergic neurotransmission. ***PLoS One.*** 2012;7(6):e39997. Epub 2012 Jun 29.

2 Chapter 2

2.1 Chapter Summary

Autonomic dysfunction is observed in many cardiovascular diseases and contributes to cardiac remodeling and heart disease. We previously reported that a decrease in the expression levels of the vesicular acetylcholine transporter (VAChT) in genetically-modified homozygous mice (VAChT KD^{HOM}) leads to decreased cholinergic tone, autonomic imbalance and a phenotype resembling heart failure. In order to further understand the molecular changes resulting from chronic long-term decrease in parasympathetic tone, we undertook a transcriptome-based, microarray-driven approach to analyze gene expression changes in ventricular tissue from VAChT KD^{HOM} mice. We demonstrate that a decrease in cholinergic tone is associated with alterations in gene expression in mutant hearts, which might contribute to increased ROS levels observed in these cardiomyocytes. In contrast, in another model of cardiac remodeling and autonomic imbalance, induced through chronic isoproterenol treatment to increase sympathetic drive, these genes did not appear to be altered in a pattern similar to that observed in VAChT KD^{HOM} hearts. These data suggest the importance of maintaining a fine balance between the two branches of the autonomic nervous system and the significance of absolute levels of cholinergic tone in proper cardiac function.

2.2 Introduction

In many cardiovascular diseases an over-activation of sympathetic tone coupled with a decrease in parasympathetic tone is observed [1-3]. This leads to an imbalance between the two branches of the autonomic nervous system (ANS) that seems to contribute to cardiac remodeling. The ANS is the main regulator of cardiac output and, as such, plays a vital role in maintaining proper heart function. Recently, we reported that a systemic reduction in the vesicular acetylcholine transporter (VAChT; [4]), the protein responsible for packaging ACh into synaptic vesicles at parasympathetic nerve terminals, leads to a phenotype resembling heart failure in mice [5]. Importantly, clinical presentation of heart failure is often associated with increased activation of various neurohumoral processes, including enhanced sympathetic activity and RAAS activation. However, due to the lack of induction of these processes in VAChT KD^{HOM} mice, they do not present with all the molecular changes observed in the failing heart. The mutant hearts have altered calcium handling and show changes in myocyte contractility, causing decreased left ventricular fractional shortening in the VAChT mutant mice [5]. Furthermore, these pathological changes can be reversed through treatment with pyridostigmine, a peripheral cholinesterase inhibitor, thus suggesting that cardiac dysfunction in these mice results from a reduction in cholinergic tone [5].

These results are in agreement with a number of recent publications which indicate that increased levels of ACh can be protective and increase survival in experimental models of heart failure. For example, vagal stimulation improves outcome in experimental models of heart failure in rats [6]. It has also been reported that chronic treatment with the cholinesterase inhibitor donepezil, an anti-Alzheimer's drug, can reduce both cardiac hypertrophy and remodeling and increase survival rates in rat and mouse models of heart failure [7, 8]. Acetylcholine, released from vagal nerve terminals, has also been shown to have cardioprotective effects through its ability to activate the phosphatidylinositol-3-kinase (PI3K)-Akt pathway [6]. Moreover, a switch to a cholinergic phenotype occurs in sympathetic terminals in mice as well as humans in heart failure and genetic inhibition of this transdifferentiation in mice worsens outcomes in experimental heart failure [9].

Hence, whereas higher levels of acetylcholine seem to be cardioprotective, lower levels are usually related to increased heart dysfunction.

In order to gain insight into potential molecular changes that may occur in the heart under conditions of chronically decreased cholinergic neurotransmission, we undertook a microarray-driven, transcriptome-based analysis of hearts from VAChT KD^{HOM} and wild-type (WT) mice. Our goal was to determine whether a long-term decrease in ACh levels leads to alterations in gene expression profiles that could contribute to heart dysfunction.

We demonstrate here that chronic reduction in cholinergic neurotransmission in VAChT mutant mice is associated with altered gene expression in the heart. Of the changes observed, increased levels of the enzyme purine nucleoside phosphorylase could contribute to increased reactive oxygen species (ROS) levels. Indeed, we found that cardiomyocytes of mutant mice present an increase in ROS. Interestingly, we demonstrate that the alterations in gene expression observed in ventricles from VAChT mutant mice are distinct from those observed in a model of cardiac dysfunction with increased sympathetic drive, indicating the importance of absolute levels of cholinergic tone in regulating cardiac function.

2.3 Materials and Methods

2.3.1 Animal models and drug administration

VAChT KD^{HOM} mice were generated as previously described [4]. Three-month old male wild-type (WT) and VAChT mutant littermates in a mixed C57BL6/j background (backcrossed for 3 generations only, as further backcrossing onto the C57BL6/j background causes infertility in this genotype) were used for all of the experiments. Mice were housed in groups of 4 per cage in a temperature-controlled room with a 12/12 light/dark cycle. Food and water were provided *ad libitum*.

For the isoproterenol infusion experiments, 3-month-old male wild-type mice from the colony were treated with isoproterenol (Sigma-Aldrich, Mississauga, Canada) at a dose of 60 mg/kg/day or saline for two weeks using the Alzet Osmotic Pumps (Model 2002, Durect Corporation, USA) and maintained in the Animal Care Facility for an additional two weeks. All animals used in these studies were maintained at the University of Western Ontario (UWO, London, Canada) and experiments were performed following the guidelines and protocols approved by the University Council on Animal Care (UCAC) for animal research.

2.3.2 RNA microarray analysis

All sample labelling and GeneChip processing was performed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). Single stranded complimentary DNA (cDNA) was prepared from 2.0 μg (ventricle extracts) of total RNA as per the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling Assay Manual (Affymetrix, Santa Clara, CA). 5.5 μg of single stranded DNA was synthesized, end labeled and hybridized, for 16 hours at 45°C, to Mouse Gene 1.0 ST arrays to probe a set of 33,000 genes. All liquid

handling steps were performed by a GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) using Command Console v1.1. Probes were summarized to gene level data in Partek Genomics Suite v6.5 (Partek, St. Louis, MO) using the RMA algorithm [10]. Partek was used to determine gene level ANOVA p-values, fold changes and GO (Gene Ontology) enrichment, using a Fisher's exact test. Differentially expressed genes were selected based on an ANOVA p-value of less than 0.05 and 1.3 fold increase or decrease between WT and knockdown samples. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE37458 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37458).

2.3.3 Quantitative RT-PCR

Total RNA was extracted using the Fatty and Fibrous Tissue RNA Extraction Kit (Bio-Rad Laboratories, Mississauga, Canada) according to the manufacturer's protocol. Total RNA from whole hearts was eluted in 80 µl of Elution Solution. Quantification and quality analysis of RNA in the extracted samples was done by microfluidic analysis using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). All RNA samples used for the reverse transcription reaction had an RNA integrity number of ≥8.0. 20 µl of cDNA was synthesized from 500 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Streetsville, Canada) following the manufacturer's instructions. cDNA was subsequently subjected to qPCR on a CFX-96 Real Time System (Biorad) using the iQ SYBR GREEN SUPERMIX (Biorad). The PCRs were cycled 40 times after initial denaturation (95^{o}C, 3 min) with the following parameters: 95^{o}C for 10 s, annealing and extension at 60^{o}C for 30s. For each experiment, a non-template reaction was used as a negative control. Relative quantification of gene expression was done with the DD*CT* method using β-actin gene expression to normalize the data. All primers used for qPCR were validated before use to confirm that efficiency was within the required range. Sequences of primers used are available upon request.

2.3.4 Immunoblotting

Whole hearts were placed in modified ice-cold RIPA buffer and homogenized using a motorized Dounce homogenizer. 40-80 μg of protein lysates were separated using SDS-PAGE and transferred onto PVDF membranes. Antibodies, dilutions and their sources are as follows: anti-acetyl-CoA carboxyase (1:1000; Cell Signaling), anti-ATP citrate lyase (1:1000; Cell Signaling), anti-fatty acid synthase (1:1000; Cell Signaling), anti-purine nucleoside phosphorylase (1:200; Santa Cruz Biotechnology), anti-α-tubulin (1:3000; Sigma-Aldrich), and anti-α-actinin (1:2000; Sigma-Aldrich). α-tubulin or α-actinin was used as a loading control for all samples and the protein was quantified using densitometry analysis.

2.3.5 Cardiomyocyte isolation

Adult ventricular myocytes were isolated as previously described [11]. Briefly, hearts were rapidly removed and perfused via the Langendorff method with Ca^{2+}-free modified Tyrode solution until the blood was cleared. Hearts were then perfused with Tyrode solution containing 100 μM $CaCl_2$ along with 1.4 mg/ml collagenase (type 2; Worthington, Lakewood, NJ) and 0.04 mg/ml protease (type XIV; Sigma, St. Louis, MO) until they were soft (~10 min). The hearts were removed from the perfusion apparatus, minced into ~1-mm chunks, and stirred for 4 min in Tyrode solution containing 0.1 mM $CaCl_2$, 0.7 mg/ml collagenase, and 0.02 mg/ml protease. Cells were filtered through a 200-μm mesh to remove tissue chunks, and extracellular Ca^{2+} concentration was raised to 0.5 mM over 10 min through three centrifuge cycles.

2.3.6 ROS measurement

MitoSOX Red (Invitrogen) was used to measure mitochondrial reactive oxygen species (ROS) production. Isolated cardiomyocytes were loaded with MitoSOX Red (3 μmol/l) in DMEM for 20 min at room temperature, followed by washout. Confocal images were obtained by excitation at 514 nm and measuring the emitted light at 585 nm in cardiomyocytes bathed in normal Tyrode's solution. The confocal imaging was

performed with a Zeiss LSM 510 META confocal microscope (CEMEL-Confocal Microscopy Facility, ICB/UFMG).

2.3.7 Statistical analyses

Results of qPCR, immunoblotting and MitoSOX experiments are provided as mean ± SEM. The student's t-test was used to assess statistical differences between two experimental groups using SigmaStat software. $p<0.05$ was considered statistically significant.

2.4 Results

2.4.1 Analysis of transcriptional alterations in VAChT KD^{HOM} hearts

We have previously demonstrated that VAChT KD^{HOM} mice with reduced cholinergic neurotransmission develop cardiac dysfunction [5]. To determine the transcriptional alterations in VAChT mutant mice, we performed a microarray analysis using total RNA isolated from ventricles obtained from VAChT KD^{HOM} and age-matched WT mice. Ventricular tissue was used for the microarray analysis since previous experiments with these mice revealed major alterations related to cardiac dysfunction in isolated ventricular cardiomyocytes [5]. Therefore, we wanted to determine whether there were any changes in gene expression in ventricular tissue that may contribute to this dysfunction. A list of differentially expressed genes was generated by limiting the fold change to at least 1.3 with a significance of $p \leq 0.05$ (Table 2.1). The gene ontology (GO) analysis did not reveal robust alterations in any specific pathway in the mutant ventricles. However, a total of 71 genes showed differential expression between VAChT KD^{HOM} and WT mice, of which 52 genes were significantly up-regulated and 19 down-regulated.

To examine the robustness of our microarray results, we performed quantitative real-time PCR (qPCR) on four randomly chosen genes which demonstrated a significant increase (Tsen15, Gas5, Kpna2, Socs4) and two genes which demonstrated a significant decrease (Rnase4, Ogdhl) in the microarray analysis. The qPCR results confirmed that the expression of Tsen15, Gas5, Kpna2 and Socs4 were significantly increased and that of Rnase4 and Ogdhl were significantly decreased in the ventricles obtained from VAChT KD^{HOM} mice (Fig. 2.1), a result which confirms our microarray data. Further analysis of the ventricular microarray suggested a significant upregulation in the expression of two purine nucleoside phosphorylases, Pnp and Pnp2. Both Pnp and Pnp2 are involved in generating hypoxanthine, a metabolite of adenosine [12]. Hypoxanthine, produced in endothelial cells, has been shown to translocate into cardiomyocytes and contribute to production of oxygen free radicals [13, 14]. Several studies have previously shown that

Table 2.1 – Genes which show transcriptional alterations in ventricles from VAChT KD^{HOM} mutants.

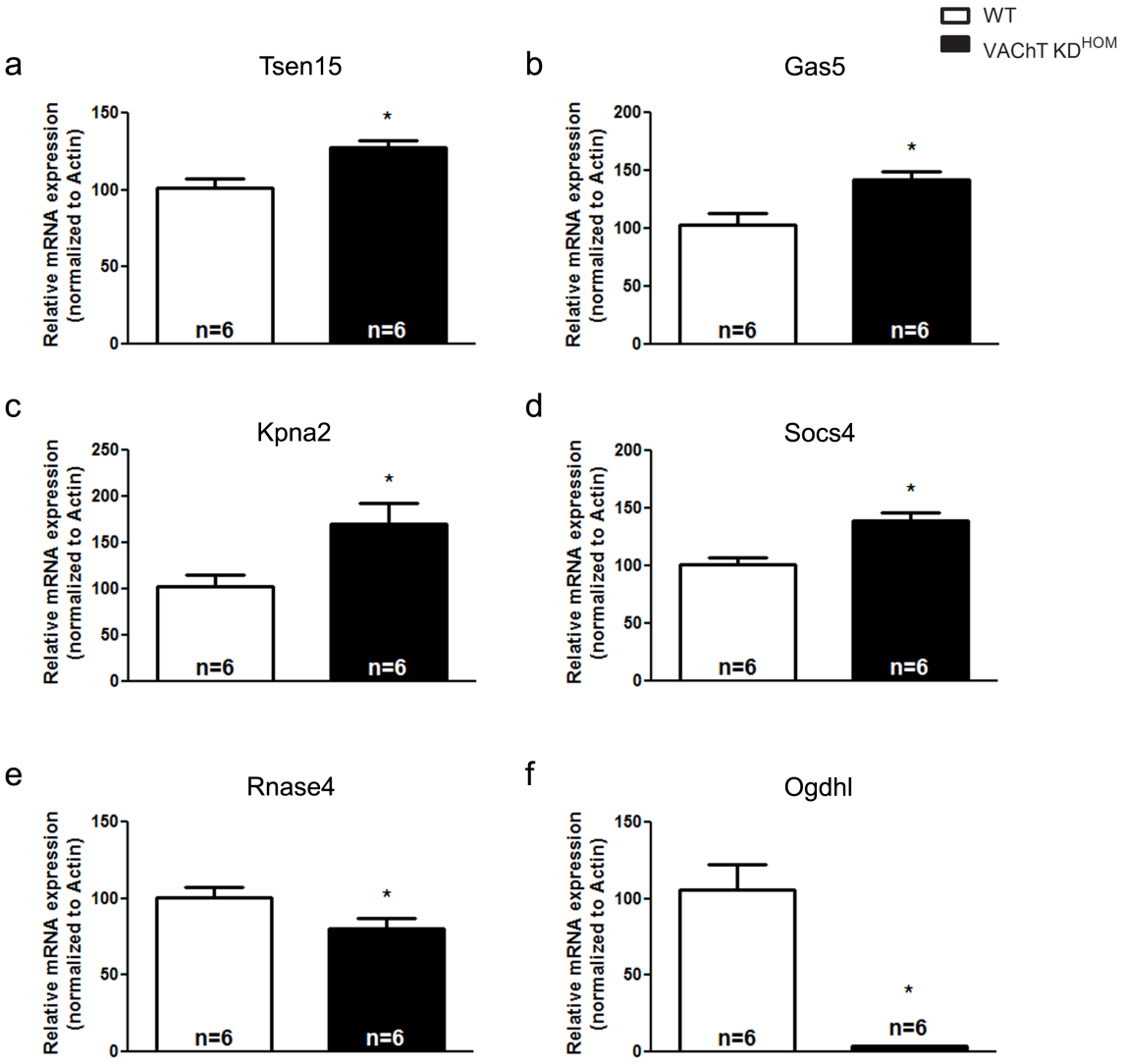
Gene Name	Gene Symbol	Transcript ID	RefSeq ID	Normalized fold-change
tRNA splicing endonuclease 15 homolog (S. cerevisiae)	Tsen15	10536593	NM_025677	1.89193
cyclin B1 interacting protein 1	Ccnb1ip1	10419465	NM_001111119	1.84595
purine-nucleoside phosphorylase	Pnp	10414514	NM_013632	1.73353
growth arrest specific 5	Gas5	10351026	NR_002840	1.58404
karyopherin (importin) alpha 2	Kpna2	10497503	NM_010655	1.56091
small nucleolar RNA, C/D box 1C	Snord1c	10382844	NR_028569	1.55143
purine-nucleoside phosphorylase 2	Pnp2	10414527	NM_001123371	1.5345
MU-2/AP1M2 domain containing, death-inducing	Mudeng	10414449	NM_144535	1.53211
ribonuclease, RNase A family, 1 (pancreatic)	Rnase1	10419563	NM_011271	1.46911
mitochondrial ribosomal protein L47	Mrpl47	10497703	NM_029017	1.43058
membrane-spanning 4-domains, subfamily A, member 6D	Ms4a6d	10466210	NM_026835	1.41185
calmodulin binding transcription activator 1	Camta1	10518835	NM_001081557	1.40688
small nucleolar RNA, C/D box 34	Snord34	10563110	NR_002455	1.40542
acyl-CoA thioesterase 10	Acot10	10427883	NM_022816	1.40355
LysM, putative peptidoglycan-binding, domain containing 2	Lysmd2	10587226	NM_027309	1.40225
suppressor of cytokine signaling 4	Socs4	10414350	NM_080843	1.39699
centrosome and spindle pole associated protein 1	Cspp1	10344817	NM_026493	1.39383
listerin E3 ubiquitin protein ligase 1	Ltn1	10440568	NM_001081068	1.39239
killer cell lectin-like receptor subfamily A, member 9	Klra9	10548511	ENSMUST00000088019	1.39002
protein tyrosine phosphatase-like A domain containing 1	Ptplad1	10594501	NM_021345	1.38888
profilin 2	Pfn2	10498309	NM_019410	1.38519
LLP homolog, long-term synaptic facilitation (Aplysia)	Llph	10515293	NM_025431	1.3812
triadin	Trdn	10362454	NM_029726	1.37467
COBW domain containing 1	Cbwd1	10466818	NR_033744	1.37322
S-adenosylmethionine decarboxylase 1	Amd1	10447510	NM_009665	1.3687
LLP homolog, long-term synaptic facilitation (Aplysia)	Llph	10603702	NM_025431	1.36867
mitochondrial ribosomal protein L20	Mrpl20	10511149	NM_025570	1.3685
phosphatidylserine decarboxylase, pseudogene 2	Pisd-ps2	10447517	NR_003519	1.36647
microRNA 208a	Mir208a	10419932	NR_029724	1.35736
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex	Ndufaf1	10486284	NM_027175	1.35388
membrane-spanning 4-domains, subfamily A, member 6B	Ms4a6b	10461622	NM_027209	1.34442

Mki67 (FHA domain) interacting nucleolar phosphoprotein	Mki67ip	10349239	NM_026472	1.34429
La ribonucleoprotein domain family, member 7	Larp7	10514383	NM_138593	1.3437
ribonuclease H2, subunit B	Rnaseh2b	10415791	NM_026001	1.34199
tubulin cofactor A	Tbca	10406710	NM_009321	1.337
FAST kinase domains 1	Fastkd1	10483521	NM_177244	1.32967
chromatin modifying protein 5	Chmp5	10504008	NM_029814	1.32433
mitochondrial ribosomal protein L22	Mrpl22	10376320	NM_175001	1.32406
SH3-binding domain glutamic acid-rich protein	Sh3bgr	10437180	NM_015825	1.32303
DCN1, defective in cullin neddylation 1, domain containing 5	Dcun1d5	10583034	NM_029775	1.31991
M phase phosphoprotein 6	Mphosph6	10478283	NM_026758	1.31718
DnaJ (Hsp40) homolog, subfamily B, member 11	Dnajb11	10434675	NM_001190804	1.3166
HIG1 domain family, member 1A	Higd1a	10597871	NM_019814	1.31585
polymerase (DNA directed), iota	Poli	10459655	NM_011972	1.31454
small nucleolar RNA, H/ACA box 26	Snora26	10522465	NR_031758	1.31066
mediator of RNA polymerase II transcription, subunit 31 homolog	Med31	10388154	NM_026068	1.31001
cAMP-regulated phosphoprotein 19	Arpp19	10587104	NM_021548	1.30743
TDP-glucose 4,6-dehydratase	Tgds	10422259	NM_029578	1.30694
FtsJ methyltransferase domain containing 1	Ftsjd1	10575376	NM_146215	1.30622
isopentenyl-diphosphate delta isomerase	Idi1	10482762	NM_145360	1.30457
transmembrane protein 19	Tmem19	10372488	NM_133683	1.3031
E2F transcription factor 6	E2f6	10394690	NM_033270	1.30278
olfactory receptor 1087	Olfr1087	10484626	NM_146846	-1.30118
vomeronasal 1 receptor 210	Vmn1r210	10408150	NM_134235	-1.30189
protease, serine-like 1	Prssl1	10370644	NM_001042710	-1.30456
microRNA 501	Mir501	10603304	NR_030496	-1.30937
ninein	Nin	10396146	NM_008697	-1.31064
LIM homeobox protein 2	Lhx2	10471833	NM_010710	-1.31587
zinc finger protein 160	Zfp160	10442177	ENSMUST00000088811	-1.31771
thymocyte selection associated	Themis	10362350	NM_178666	-1.3193
olfactory receptor 229	Olfr229	10584516	NM_146613	-1.31993
angiopoietin-like 4	Angptl4	10450038	NM_020581	-1.32425
taste receptor, type 2, member 115	Tas2r115	10548665	NM_207020	-1.32888
3-phosphoglycerate dehydrogenase	Phgdh	10500529	NM_016966	-1.32932
transmembrane protein 121	Tmem121	10399021	NM_153776	-1.34042

neuropilin (NRP) and tolloid (TLL)-like 1	Neto1	10457091	NM_144946	-1.34581
cyclin M2	Cnnm2	10468249	NM_033569	-1.3752
peripherin 2	Prph2	10445633	NM_008938	-1.38184
ribonuclease, RNase A family 4	Rnase4	10414537	NM_021472	-1.4077
late cornified envelope 1C	Lce1c	10493889	NM_028622	-1.42251
oxoglutarate dehydrogenase-like	Ogdhl	10413874	NM_001081130	-7.53128

Figure 2.1 - qPCR analysis confirms the expression pattern of several genes detected in the microarray analysis. mRNA expression of tRNA splicing endonuclease 3 (Tsen15; panel a), Growth arrest specific 5 (Gas5; panel b), karyopherin alpha 2 (Kpna2; panel c) and suppressor of cytokine signaling 4 (Socs4; panel d) was increased in mutant mice. mRNA analysis of ribonuclease, RNase A family 4 (Rnase4; panel e) and oxoglutarate-dehydrogenase like (Ogdhl; panel f) confirmed transcriptional downregulation in whole heart RNA from the mutant animals. Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.1 - qPCR analysis confirms the expression pattern of several genes detected in the microarray analysis.



increased ROS levels can play a major role in cardiac dysfunction in different cardiomyopathies [15-18]. Therefore, due to the potential role of these enzymes in contributing to cardiac malfunction in VAChT KD^{HOM} mice, the increased expression of these enzymes was further confirmed via qPCR (Fig. 2.2a,b). Importantly, this transcriptional upregulation in Pnp/Pnp2 led to a significant increase in the protein content of Pnp/Pnp2 in ventricular tissues obtained from VAChT mutant mice (Fig. 2.2c).

2.4.2 Mitochondrial superoxide levels are increased in VAChT KD^{HOM} animals

The increased expression of Pnp/Pnp2 in the hearts of mutant mice might predict a greater production of ROS (as determined via superoxide formation) in these mice. Interestingly, cardiomyocytes from VAChT KD^{HOM} hearts showed a significant increase in superoxide levels as compared to wild-type control cells (Fig. 2.3), suggesting greater levels of ROS production in VAChT KD cardiomyocytes as compared to WT mice.

2.4.3 Lipid biosynthesis appears to be unaltered in VAChT KD^{HOM} mice

In addition to changes in ROS, which may contribute to phenotypic changes in these mice, previous work has suggested a decrease in fatty acid oxidation during heart failure can also contribute to further decline in cardiac function [19-22]. In order to examine other potential changes in transcripts that could affect cardiac energetics in the VAChT mutant mice, we used a candidate gene approach and analyzed specific genes involved in the lipid biosynthetic process. In particular, we studied the expression of ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). These genes, related to cardiac metabolism, are known to be altered in severe heart failure [23]. ACLY is responsible for converting citrate into oxaloacetate in the cytoplasm and, in the process, generates a molecule of Acetyl-CoA which is utilized by ACC to produce Malonyl-CoA, the substrate used for the generation of long chain fatty acids by FAS. Real-time PCR revealed a significant upregulation in the expression of all three of these

Figure 2.2 - Purine nucleoside phosphorylases are upregulated in the hearts of VAChT KD^{HOM} mice. mRNA expression of purine nucleoside phosphorylase (Pnp; **panel a**) and purine nucleoside phosphorylase 2 (Pnp2, **panel b**) were upregulated. Pnp/Pnp2 protein content appears to upregulated in VAChT KD^{HOM} animals as compared to wild-type mice (**panel c**). Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.2 - Purine nucleoside phosphorylases are upregulated in the hearts of VAChT KD^{HOM} mice.

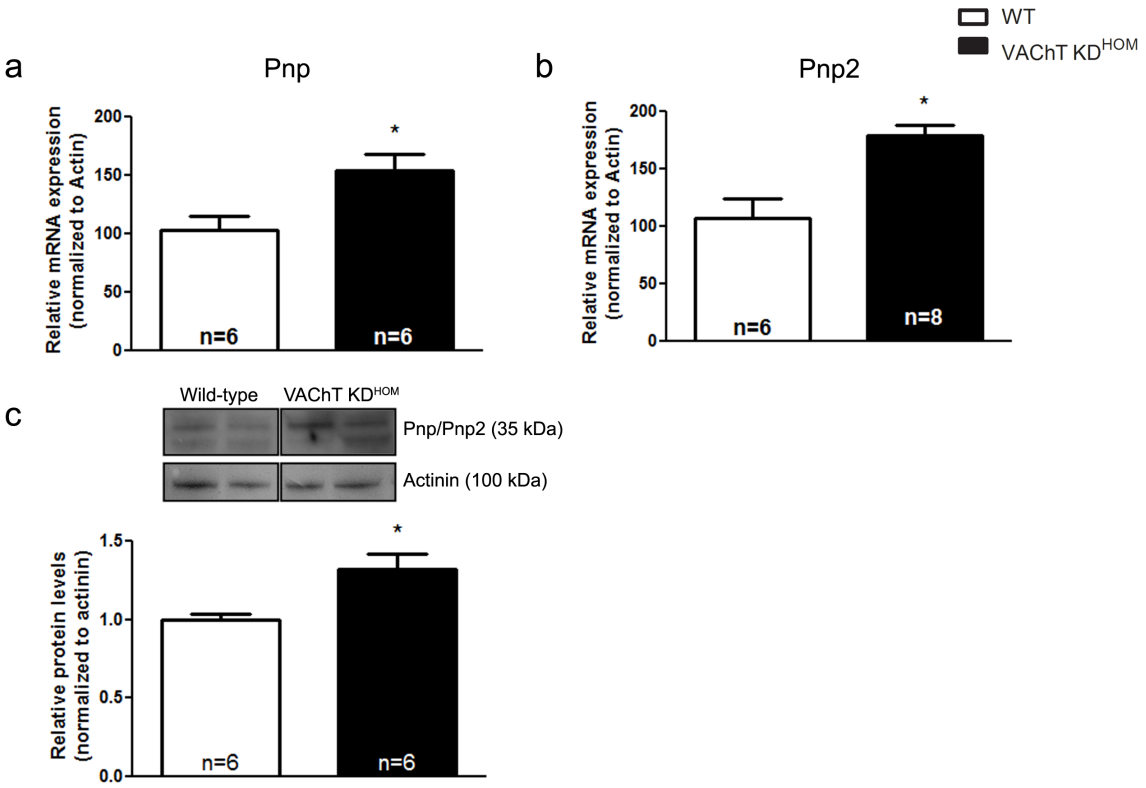


Figure 2.3 - VAChT KD^{HOM} cardiomyocytes show increased levels of ROS. Isolated cardiomyocytes loaded with a MitoSOX superoxide indicator reveal greater ROS levels in mutant myocytes (sample image; **panel a**). A robust, significant increase in fluorescence was observed in the KD cardiomyocytes as compared to wild-type control cells (**panel b**). Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice. Scale bar=10μm.

Figure 2.3 - VAChT KD^{HOM} cardiomyocytes show increased levels of ROS.

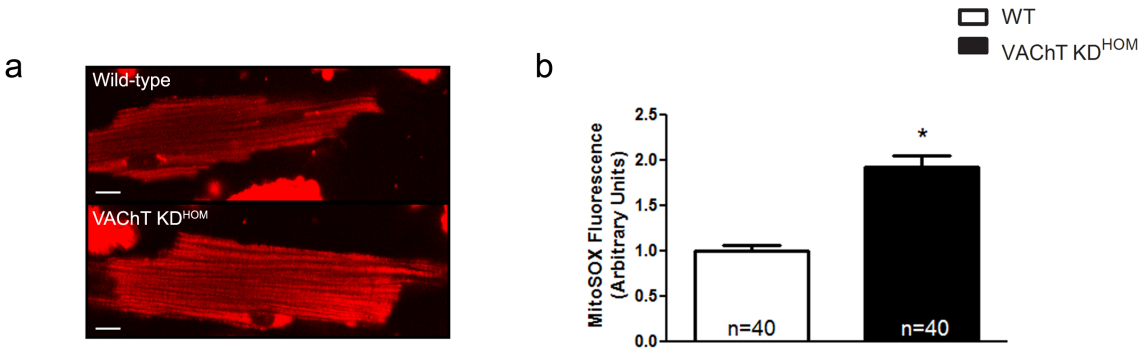
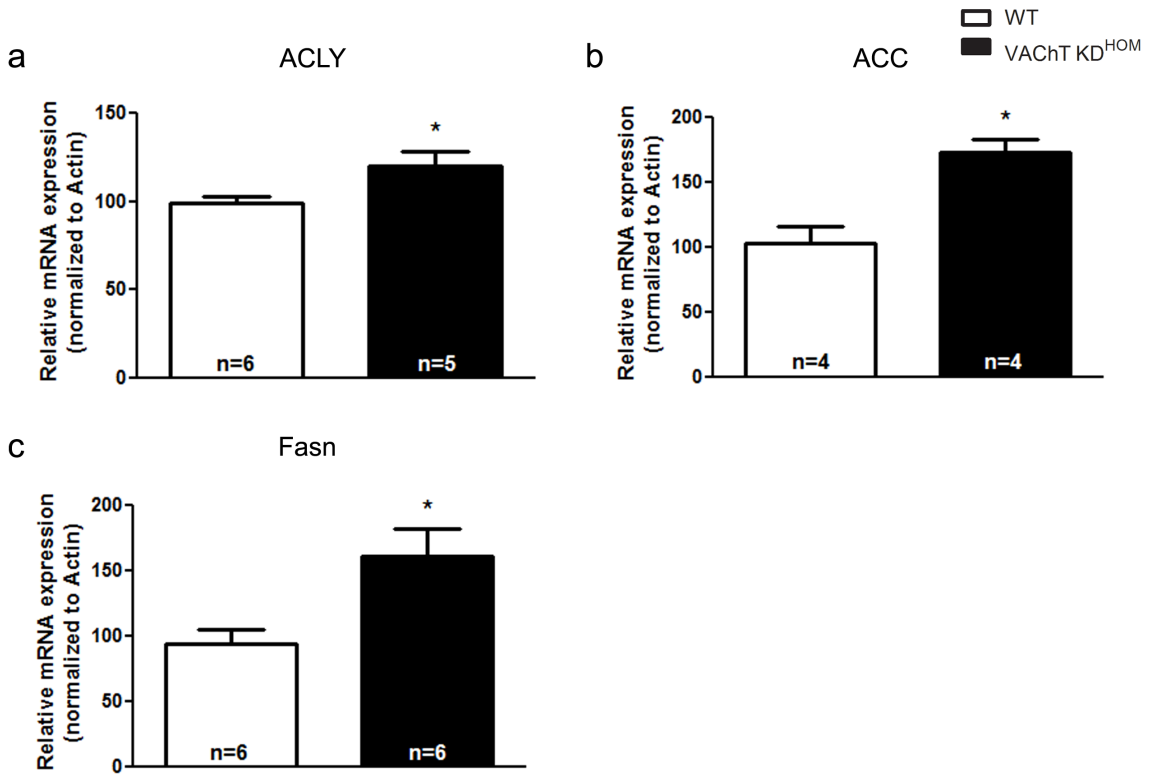


Figure 2.4 - The transcription of genes related to fatty acid biosynthesis is upregulated. mRNA expression of ATP citrate lyase (ACLY, **panel a**), Acetyl-CoA carboxylase (ACC; **panel b**) and fatty acid synthase (FAS; **panel c**) was increased expression in VAChT KD^{HOM} mice. Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.4 - The transcription of genes related to fatty acid biosynthesis is upregulated.



genes in VAChT-mutant mice (Fig. 2.4). However, assessment of protein expression levels using immunoblotting revealed no significant differences in the protein levels of these enzymes between WT and VAChT mutant mice (Fig. 2.5).

2.4.4 Analysis of transcriptional alterations in isoproterenol-treated hearts

It is unclear whether autonomic imbalance due to reduced cholinergic tone has similar consequences to imbalance due to increased sympathetic tone. To examine whether the alterations in gene expression observed in the ventricles of VAChT KD^{HOM} mice were observed in a different model of cardiac dysfunction, we chronically treated wild-type mice with isoproterenol to induce cardiac remodeling. Isoproterenol-treated mice demonstrated a significant increase in heart weight to tibia length ratio as compared to saline-treated mice (Fig. 2.6a). Furthermore, isoproterenol-treated mice demonstrated a significant increase in the expression of two markers of cardiac stress; namely, β-myosin heavy chain (β-MHC: Fig. 2.6b) and atrial natriuretic peptide (ANP; Fig. 2.6c). Next we examined potential changes in the expression pattern of genes that displayed altered expression in the VAChT KD^{HOM} mice. Interestingly, most of the genes which were altered in VAChT KD^{HOM} mice were not significantly altered in hearts obtained from isoproterenol-treated mice (Fig. 2.7a-e). However, the expression of Ogdhl (Fig. 2.7f) was significantly decreased following chronic treatment with isoproterenol, similar to the transcriptional alterations observed in the VAChT mutant mice. The expression of PnP and Pnp2 were not significantly different between isoproterenol-treated and saline-treated mice (Fig. 2.8a,b), suggesting that alterations in these genes are specifically related to a decrease in cholinergic tone.

In contrast to the transcriptional changes found in the microarray experiments, the expression of genes related to the lipid biosynthetic pathway did, in fact, appear to be similarly altered in isoproterenol-treated mice and VAChT KD^{HOM} mice (Fig. 2.8c,d,e). Furthermore, the relative change in the expression of these genes was much larger in isoproterenol-treated mice than that observed in VAChT KD^{HOM} hearts. Hence, some, but

not all transcriptional alterations are reproduced between isoproterenol-treated mice and VAChT KD^{HOM} mice.

Figure 2.5 - There are no alterations in the protein levels of enzymes involved in lipid biosynthesis. Immunoblotting analysis of ATP citrate lyase (ACLY, **panel a**), Acetyl-CoA carboxylase (ACC; **panel b**) and fatty acid synthase (FAS; **panel c**) revealed no differences in the protein levels of these enzymes in VAChT KD^{HOM} mice. Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.5 - There are no alterations in the protein levels of enzymes involved in lipid biosynthesis.

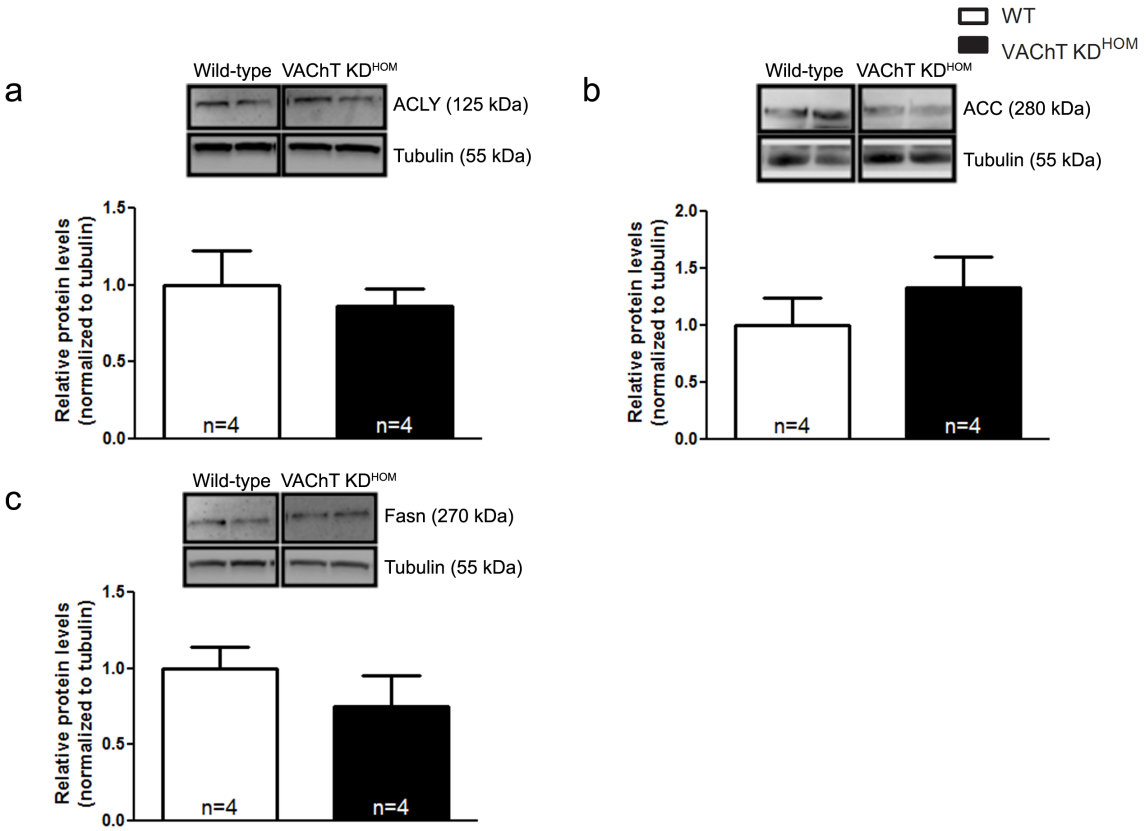


Figure 2.6 - Chronic treatment with isoproterenol (ISO)-induces cardiac remodeling in wild-type mice. Two week ISO treatment led to a significant increase in heart weight/tibia length ratio as compared to saline-treated control mice (**panel a**). The expression of β-myosin heavy chain (β-MHC, **panel b**) and atrial natriuretic factor (ANF, **panel c**) were significantly upregulated in ISO-treated mice. Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.6 - Chronic treatment with isoproterenol (ISO)-induces cardiac remodeling in wild-type mice.

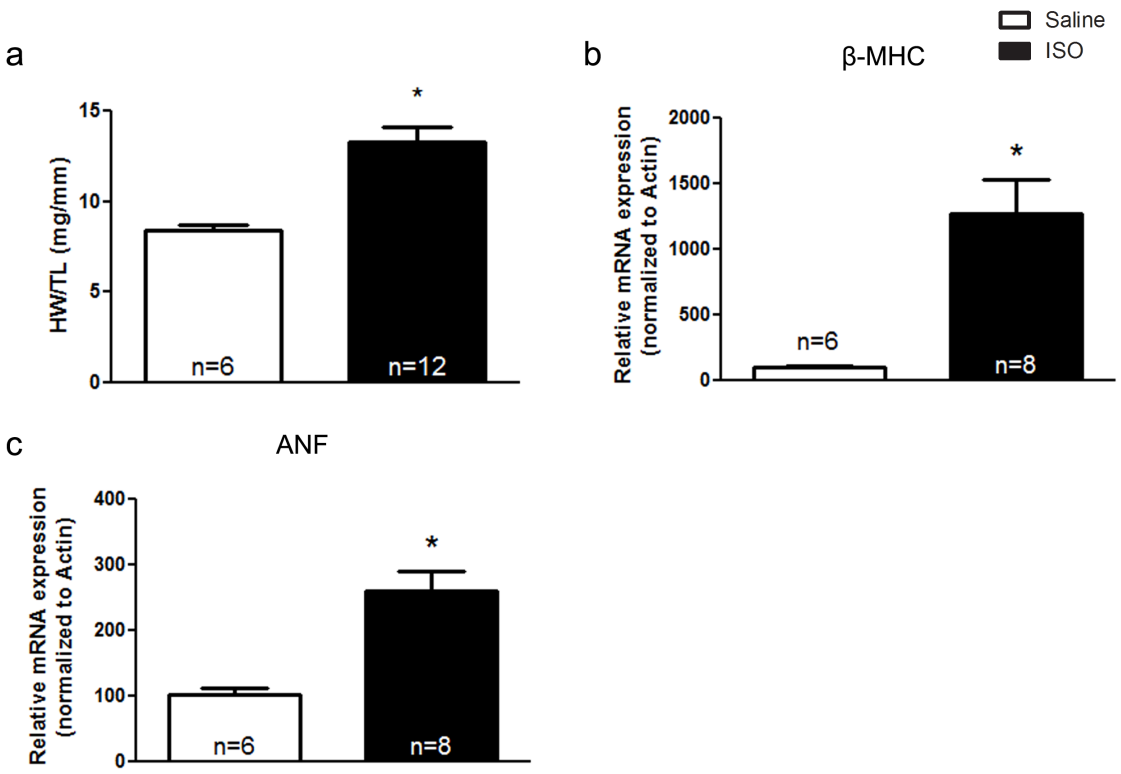


Figure 2.7 - ISO treatment does not lead to the same transcriptional alterations observed in VAChT KD^{HOM} mice. mRNA expression of tRNA splicing endonuclease 3 (Tsen15; **panel a**), Growth arrest specific 5 (Gas5; **panel b**), karyopherin alpha 2 (Kpna2; **panel c**), suppressor of cytokine signaling 4 (Socs4; **panel d**), ribonuclease, RNase A family 4 (Rnase4; **panel e**) and oxoglutarate-dehydrogenase like (Ogdhl; **panel f**) were not significantly altered in the ISO-treated mice as compared to saline-treated controls. Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.7 - ISO treatment does not lead to the same transcriptional alterations observed in VAChT KD^{HOM} mice.

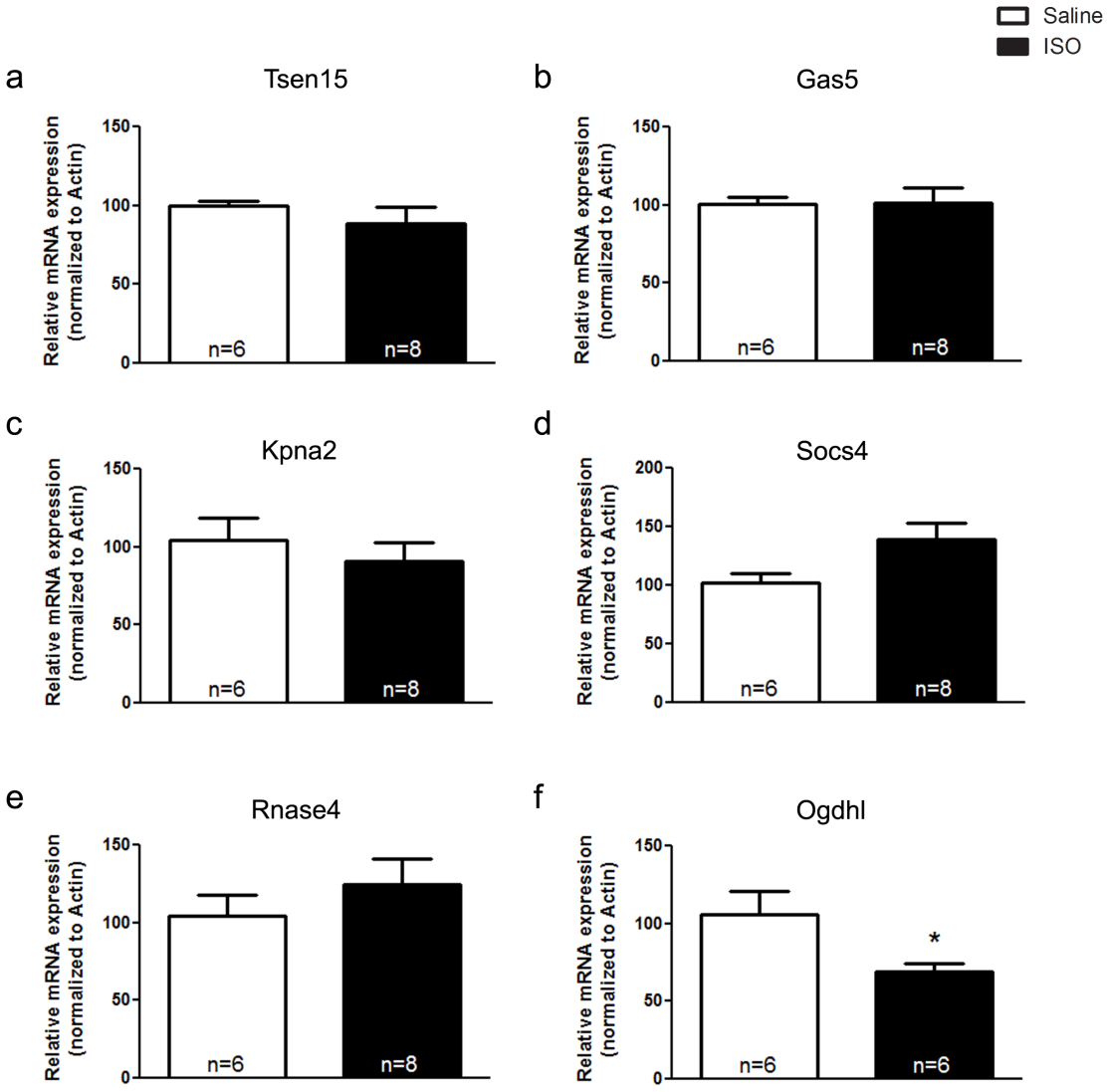
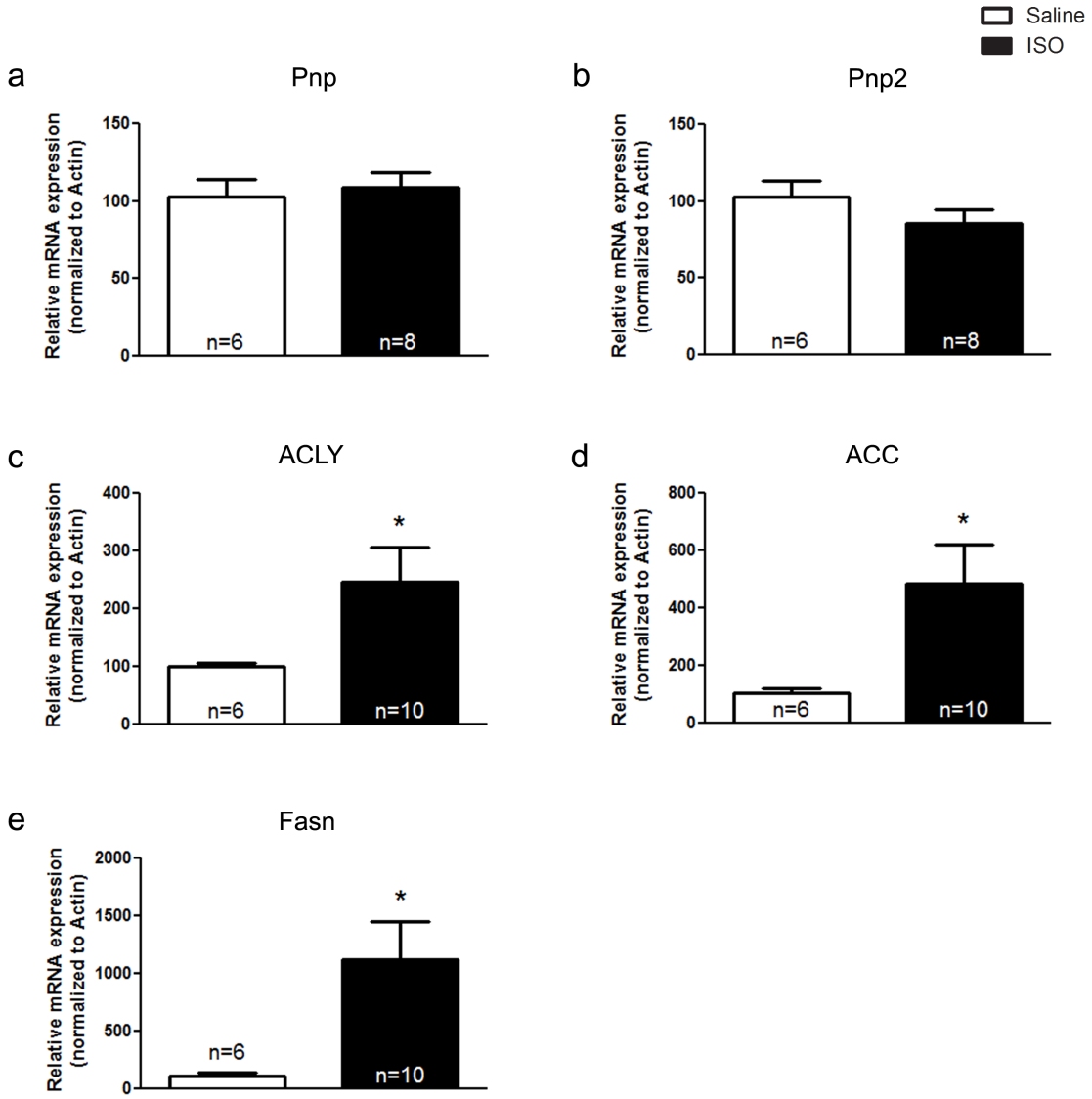


Figure 2.8 - Expression of genes related to fatty acid synthesis is increased following isoproterenol (ISO) treatment. mRNA expression of purine nucleoside phosphrylases (Pnp and Pnp2; **panels a** and **panel b**) were not altered following ISO treatment. ATP citrate lyase (ACLY; **panel c**), acetyl-CoA carboxylase (ACC; **panel d**) and fatty acid synthase (FAS; **panel e**) expression were all significantly increased in ISO-treated mice as compared to saline-treated controls. Data represent the mean ± SEM, with n indicated within bars. *$p<0.05$ versus wild-type mice.

Figure 2.8 - Expression of genes related to fatty acid synthesis is increased following isoproterenol (ISO) treatment.



2.5 Discussion

Recent reports have implicated the parasympathetic nervous system in the development and progression of heart failure as well as being a potential therapeutic target in heart disease [24]. Vagal stimulation can improve outcome in experimental heart failure [6] and this can be mimicked by treatment with cholinesterase inhibitors [8]. Our previous studies have demonstrated that mice with a systemic decrease in cholinergic tone develop cardiac dysfunction and exhibit many of the characteristics present in cardiac remodeling [5]. Importantly, in VAChT KD^{HOM} mice, these cardiac defects are ameliorated by cholinesterase inhibitor treatment, implicating release of ACh rather than developmentally-induced changes in the control of heart function. Interestingly, in heart failure, transdifferentiation of sympathetic neurons into a cholinergic phenotype has recently been demonstrated, and this appears to play a protective role [9]. However, the role of acetylcholine in controlling long-term cardiac function is still poorly understood.

The present study examined whether the decrease in parasympathetic tone in VAChT KD^{HOM} mice leads to alterations in cardiac gene expression, which may contribute to the observed cardiac dysfunction. Our microarray analysis revealed a number of transcriptional changes with a total of 71 genes being significantly different between WT and KD mice. Interestingly, transcript and protein levels of two purine nucleoside phosphorylases (Pnp and Pnp2) were significantly increased in the VAChT mutant mice. Both Pnp and Pnp2 are important enzymes responsible for the conversion of inosine to hypoxanthine and have also been shown to metabolize adenosine into adenine, especially under conditions of cardiac stress [12, 25, 26].

The increased levels of Pnp and Pnp2 in VAChT KD^{HOM} mice may lead to the increased production of hypoxanthine in mutant hearts [12]. Interestingly, endothelial cells in the heart appear to be responsible for the majority of adenosine uptake [27]. In addition, they are responsible for the metabolism of adenosine into several compounds, including hypoxanthine [28]. Increased levels of this adenosine metabolite may serve a key role in

the cardiac dysfunction observed in mutant mice. Hypoxanthine, for example, is produced in endothelial cells and taken up by the ENT and ENBT1 transporters into myocytes, where it is further metabolized into xanthine and urate [13, 14]. These metabolites contribute to the production of ROS, which have been shown to play a role in cardiac and vascular dysfunction in both ischemic and non-ischemic cardiomyopathies [15-18]. Interestingly, we observed an increase in the levels of ROS in ventricular cardiomyocytes isolated from VAChT KD^{HOM} mice. Oxygen free radicals contribute to declining cardiac function during heart failure via many different mechanisms and result in damage to the myocardium [29, 30]. They can also have detrimental effects specifically in cardiomyocytes as they can activate cell death through both necrotic and apoptotic pathways [31, 32]. Future studies will be necessary to further characterize the mechanisms which lead to increased ROS production in the mutant mice as well as determine the physiological importance of these oxygen free radicals and their role in the observed cardiac dysfunction. However, it is tempting to speculate that these alterations may play a role in the dysfunction found in VAChT mutant mice.

It is important to note that ADP levels are increased in heart failure suggesting that failing cardiac tissue utilizes greater amounts of ATP [33]. Under normal conditions, the vasodilatory actions of adenosine may be able to compensate for this increased utilization of ATP by myocardial tissue. However, VAChT KD^{HOM} mice show increased levels of Pnp which has previously been shown to metabolize adenosine into adenine [25, 26]. This may contribute to the inability of the mutant hearts to maintain normal contractile function, an idea which is in accordance with previous research suggesting that the failing heart is energy-starved [34, 35]. It should be noted that these alterations seems to be selectively related to decreased cholinergic function, as the changes observed in the microarray experiments were not observed in isoproterenol-treated mice.

Significant alterations in substrate metabolism have been observed during the progression of heart failure and it is suggested that these changes contribute to cardiac remodeling and dysfunction observed during disease progression [36]. Previous studies have shown that, in end stage heart failure, there is an increase in glucose oxidation coupled with a

decrease in fatty acid oxidation and these changes in substrate utilization lead to adverse effects during late stage heart failure [19-22].

To further examine transcriptional alterations in VAChT mutant hearts that may not have been identified in the microarray, we chose genes related to the lipid biosynthetic pathway (ACLY, ACC and FAS). These pathways have been previously found to be altered in heart failure [23]. In agreement with the notion that these pathways may be altered in cardiac dysfunction, chronic treatment with the β-agonist isoproterenol, which mimics the sympathetic overactivation observed in several cardiac diseases, increased mRNA levels for ACLY, ACC and FAS several fold. We also found an increase in mRNA expression of ACLY, ACC and FAS in VAChT mutant mice, suggesting at least some similarities between autonomic imbalance due to decreased cholinergic tone and sympathetic overactivation. Although gene expression changes were confirmed for several genes involved in the generation of long-chain fatty acids in VAChT-mutant mice, the protein levels for these enzymes appeared to be unaltered, although we cannot discard the possibility that their turnover might be increased.

It is important to recognize that the transcriptional differences observed in ISO-treated hearts is distinct from those observed in VAChT KD^{HOM} mice. It is possible that at least some of these differences are due to the genetic mouse strain utilized for the ISO treatment experiment. It has long been recognized that the genetic divergence between common laboratory mouse strains leads to changes in their response to cardiac stress. As such, a more detailed study will be required to elucidate the basis of the transcriptional differences observed in VAChT KD^{HOM} and ISO-treated mice to determine which alterations are due to a difference in mouse strains, as opposed to changes in autonomic signaling.

An additional caveat must be taken into consideration when comparing the ISO model of cardiac dysfunction with VAChT KD^{HOM} animals. The extent of cardiac remodeling in both of these models has not been thorough analyzed and, as such, there is no evidence that the extent of cardiac remodeling is similar in both animal models. It is likely that a

difference in the extent of molecular remodeling may contribute to the altered transcriptional response observed when the two models are compared.

Importantly, VAChT KD^{HOM} animals exhibit a global decrease in VAChT levels and, therefore, decreased cholinergic tone. This is significant because it has recently been proposed that cardiomyocytes possess the machinery (VAChT, ChAT and CHT1) for *de novo* production of ACh [37, 38] and are able to synthesize and release this neurotransmitter. This non-neuronal ACh may then act in an autocrine/paracrine fashion to amplify neuronal cholinergic signaling [38]. We have found that this non-neuronal cardiomyocyte release of ACh plays an important role in the protection of myocytes against isoproterenol-induced hypertrophy and that VAChT mutant mice are deficient in non-neuronal ACh secretion as well [39]. We cannot discard the possibility that the gene alterations we uncovered here may, at least in part, be due to deficient ACh release from cardiomyocytes. Future studies will be necessary to specifically analyze the importance of this non-neuronal cholinergic system in myocytes and its contribution to the cardiac dysfunction observed after reduced cholinergic tone. This may provide an unanticipated mechanism by which non-neuronal ACh can play an important role in cardiac function.

2.6 Acknowledgments

This work was supported by the Heart and Stroke Foundation of Ontario (grant NA 6656), Canadian Institutes for Health Research (grant MOP-82756 and MOP-89919), NIH R03TW008425 from the Fogarty International Center (SG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (SG) and Programa de Apoio a Núcleos de Excelência (PRONEX-CBB APQ-04334-10 and APQ-00746-09). A.R. is supported by the Queen Elizabeth II Graduate Scholarship in Science and Technology (QEIIGSST). A.L. is a recipient of the CNPq PhD fellowship at the Post-graduation Program in Biological Science: Physiology and Pharmacology at UFMG. D.G. is a PhD student at the Graduate Program Santa Casa, Belo Horizonte, MG, Brazil. R.G. is supported by a New Investigator Award from the Heart and Stroke Foundation of Canada.

2.7 References

[1] Hasking GJ, Esler MD, Jennings GL, Burton D, Johns JA, Korner PI. Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity. Circulation. 1986;73:615-21.

[2] Nolan J, Flapan AD, Capewell S, MacDonald TM, Neilson JM, Ewing DJ. Decreased cardiac parasympathetic activity in chronic heart failure and its relation to left ventricular function. Br Heart J. 1992;67:482-5.

[3] Floras JS. Clinical aspects of sympathetic activation and parasympathetic withdrawal in heart failure. J Am Coll Cardiol. 1993;22:72A-84A.

[4] Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, et al. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron. 2006;51:601-12.

[5] Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, et al. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol. 2010;30:1746-56.

[6] Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation. 2004;109:120-4.

[7] Okazaki Y, Zheng C, Li M, Sugimachi M. Effect of the cholinesterase inhibitor donepezil on cardiac remodeling and autonomic balance in rats with heart failure. J Physiol Sci. 2010;60:67-74.

[8] Handa T, Katare RG, Kakinuma Y, Arikawa M, Ando M, Sasaguri S, et al. Anti-Alzheimer's drug, donepezil, markedly improves long-term survival after chronic heart failure in mice. J Card Fail. 2009;15:805-11.

[9] Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. The Journal of clinical investigation. 2010;120:408-21.

[10] Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 2003;31:e15.

[11] Guatimosim S, Sobie EA, dos Santos Cruz J, Martin LA, Lederer WJ. Molecular identification of a TTX-sensitive Ca(2+) current. Am J Physiol Cell Physiol. 2001;280:C1327-39.

[12] Arch JR, Newsholme EA. The control of the metabolism and the hormonal role of adenosine. Essays Biochem. 1978;14:82-123.

[13] Ward JL, Sherali A, Mo ZP, Tse CM. Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine. J Biol Chem. 2000;275:8375-81.

[14] Bone DB, Choi DS, Coe IR, Hammond JR. Nucleoside/nucleobase transport and metabolism by microvascular endothelial cells isolated from ENT1-/- mice. Am J Physiol Heart Circ Physiol. 2010;299:H847-56.

[15] Kinugasa Y, Ogino K, Furuse Y, Shiomi T, Tsutsui H, Yamamoto T, et al. Allopurinol improves cardiac dysfunction after ischemia-reperfusion via reduction of oxidative stress in isolated perfused rat hearts. Circ J. 2003;67:781-7.

[16] Sommerschild HT, Kirkeboen KA. Adenosine and cardioprotection during ischaemia and reperfusion--an overview. Acta Anaesthesiol Scand. 2000;44:1038-55.

[17] Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. J Physiol. 2004;555:589-606.

[18] Yamamoto M. Studies on free radical formation and respiratory function in mitochondria from experimentally-induced failing dog heart. Jpn Circ J. 1978;42:1320-2, 85-93.

[19] Katz AM. Metabolism of the failing heart. Cardioscience. 1993;4:199-203.

[20] Davila-Roman VG, Vedala G, Herrero P, de las Fuentes L, Rogers JG, Kelly DP, et al. Altered myocardial fatty acid and glucose metabolism in idiopathic dilated cardiomyopathy. J Am Coll Cardiol. 2002;40:271-7.

[21] Osorio JC, Stanley WC, Linke A, Castellari M, Diep QN, Panchal AR, et al. Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor-alpha in pacing-induced heart failure. Circulation. 2002;106:606-12.

[22] Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP. Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. Circulation. 1996;94:2837-42.

[23] Olson RE, Schwartz WB. Myocardial metabolism in congestive heart failure. Medicine (Baltimore). 1951;30:21-41.

[24] Olshansky B, Sabbah HN, Hauptman PJ, Colucci WS. Parasympathetic nervous system and heart failure: pathophysiology and potential implications for therapy. Circulation. 2008;118:863-71.

[25] Zimmerman TP, Gersten N, Miech RP. Adenine and Adenosine Metabolism in Liver. P Am Assoc Canc Res. 1970;11:87-&.

[26] Deuticke B, Gerlach E, Dierkesmann R. [Decomposition of free nucleotides in the rat heart, skeletal muscle, brain and liver in oxygen deficiency]. Pflugers Arch Gesamte Physiol Menschen Tiere. 1966;292:239-54.

[27] Nees S, Herzog V, Becker BF, Bock M, Des Rosiers C, Gerlach E. The coronary endothelium: a highly active metabolic barrier for adenosine. Basic Res Cardiol. 1985;80:515-29.

[28] Gerlach E, Nees S, Becker BF. The vascular endothelium: a survey of some newly evolving biochemical and physiological features. Basic Res Cardiol. 1985;80:459-74.

[29] Singal PK, Khaper N, Palace V, Kumar D. The role of oxidative stress in the genesis of heart disease. Cardiovasc Res. 1998;40:426-32.

[30] Shizukuda Y, Buttrick PM. Oxygen free radicals and heart failure: new insight into an old question. Am J Physiol Lung Cell Mol Physiol. 2002;283:L237-8.

[31] Hunt MJ, Aru GM, Hayden MR, Moore CK, Hoit BD, Tyagi SC. Induction of oxidative stress and disintegrin metalloproteinase in human heart end-stage failure. Am J Physiol Lung Cell Mol Physiol. 2002;283:L239-45.

[32] Chesley A, Lundberg MS, Asai T, Xiao RP, Ohtani S, Lakatta EG, et al. The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase. Circ Res. 2000;87:1172-9.

[33] Zhang J, Ugurbil K, From AH, Bache RJ. Use of magnetic resonance spectroscopy for in vivo evaluation of high-energy phosphate metabolism in normal and abnormal myocardium. J Cardiovasc Magn Reson. 2000;2:23-32.

[34] van Bilsen M, Smeets PJ, Gilde AJ, van der Vusse GJ. Metabolic remodelling of the failing heart: the cardiac burn-out syndrome? Cardiovasc Res. 2004;61:218-26.

[35] Katz AM. Is the failing heart energy depleted? Cardiol Clin. 1998;16:633-44, viii.

[36] Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. Physiol Rev. 2005;85:1093-129.

[37] Rana OR, Schauerte P, Kluttig R, Schroder JW, Koenen RR, Weber C, et al. Acetylcholine as an age-dependent non-neuronal source in the heart. Auton Neurosci. 2010;156:82-9.

[38] Kakinuma Y, Akiyama T, Sato T. Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J. 2009;276:5111-25.

[39] Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, de Morais Gomes ER, et al. Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. Journal of molecular and cellular cardiology. 2012;53:206-16.

Chapter 3

NON-NEURONAL CHOLINERGIC MACHINERY PRESENT IN CARDIOMYOCYTES OFFSETS HYPERTROPHIC SIGNALS

A version of this chapter has previously been published: Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, de Morais Gomes ER, Prado VF, Gros R, Guatimosim C, Prado MA, Guatimosim S. (2012). Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. ***Journal of Molecular and Cellular Cardiology.*** 2012 Aug;53(2):206-16. Epub 2012 May 14.

3 Chapter 3

3.1 Copyright Permission

ELSEVIER LICENSE
TERMS AND CONDITIONS

Jun 04, 2014

This is a License Agreement between Ashbeel Roy ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Ashbeel Roy
Customer address	100 Perth Drive London, ON N6A 5B7
License number	3401990886277
License date	Jun 04, 2014
Licensed content publisher	Elsevier
Licensed content publication	Journal of Molecular and Cellular Cardiology
Licensed content title	Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals
Licensed content author	Cibele Rocha-Resende,Ashbeel Roy,Rodrigo Resende,Marina S. Ladeira,Aline Lara,Enéas Ricardo de Morais Gomes,Vania F. Prado,Robert Gros,Cristina Guatimosim,Marco A.M. Prado,Silvia Guatimosim
Licensed content date	August 2012
Licensed content volume number	53
Licensed content issue number	2
Number of pages	11
Start Page	206
End Page	216
Type of Use	reuse in a thesis/dissertation
Portion	full article
Format	both print and electronic
Are you the author of this Elsevier article?	Yes

3.2 Chapter Summary

Recent work has provided compelling evidence that increased levels of acetylcholine (ACh) can be protective in heart failure, whereas reduced levels of ACh secretion can cause heart malfunction. Previous data show that cardiomyocytes themselves can actively secrete ACh, raising the question of whether this cardiomyocyte derived ACh may contribute to the protective effects of ACh in the heart. To address the functionality of this non-neuronal ACh machinery, we used cholinesterase inhibitors and a siRNA targeted to AChE (acetylcholinesterase) as a way to increase the availability of ACh secreted by cardiac cells. By using nitric oxide (NO) formation as a biological sensor for released ACh, we showed that cholinesterase inhibition increased NO levels in freshly isolated ventricular myocytes and that this effect was prevented by atropine, a muscarinic receptor antagonist, and by inhibition of ACh synthesis or vesicular storage. Functionally, cholinesterase inhibition prevented the hypertrophic effect as well as molecular changes and calcium transient alterations induced by adrenergic overstimulation in cardiomyocytes. Moreover, inhibition of ACh storage or atropine blunted the anti-hypertrophic action of cholinesterase inhibition. Altogether, our results show that cardiomyocytes possess functional cholinergic machinery that offsets deleterious effects of hyperadrenergic stimulation. In addition, we show that adrenergic stimulation upregulates expression levels of cholinergic components. We propose that this cardiomyocyte cholinergic signaling could amplify the protective effects of the parasympathetic nervous system in the heart and may counteract or partially neutralize hypertrophic adrenergic effects.

3.3 Introduction

Extrinsic control of heart function is primarily regulated by the autonomic nervous system. The parasympathetic branch, which releases the neurotransmitter acetylcholine (ACh), is well known to control heart rate [1] by predominantly regulating atrial function [2]. Rich cholinergic innervations are found in the sinoatrial node, atrial myocardium, atrioventricular node and in the ventricular conducting system of many species [3]. Although less abundant, parasympathetic fibers are also found throughout the ventricles, where stimulation of type 2 muscarinic acetylcholine receptor (M_2-AChR) by ACh leads to L-type calcium channel inhibition and, consequently, reduced cardiomyocyte contractility [4]. Recently, novel physiological functions of ACh in the heart have emerged and new data suggest that this neurotransmitter plays unanticipated long-term roles in cardiac protection that may not be necessarily linked to its role in regulating the electrical properties of the heart [5].

ACh is synthesized in the cytoplasm of nerve terminals through the action of choline acetyltransferase (ChAT) and stored within acidic synaptic vesicles for release. Activity coupled transport of ACh into synaptic vesicles in nerve endings is mediated by the vesicular acetylcholine transporter (VAChT) [6-8]. Released ACh, which activates muscarinic receptors on cardiac cells, is rapidly degraded by the enzyme acetylcholinesterase (AChE) to form acetate and choline. Choline is then recycled through the action of the high-affinity choline transporter (CHT1), and reused as a substrate for the synthesis of new ACh molecules [9].

Previously, we have shown that VAChT knockdown homozygous mice (VAChT KD^{HOM} mice), which express only 30% of normal VAChT levels, present a number of cholinergic deficits due to reduced ACh release [8], including a remarkable dysfunction in ventricular myocytes [10]. This is characterized by depressed ventricular contractile function, calcium (Ca^{2+}) signaling dysfunction and altered gene expression in ventricular myocytes [10]. Confirming these findings, another model of cholinergic dysfunction with reduced CHT1 expression showed age-dependent ventricular dysfunction [11]. Along

these lines, M_2-AChR knockout (KO) mice presented increased susceptibility to cardiac stress [12]. Taken together, these results suggest that decreased cholinergic function can affect outcomes for proper heart activity. Complementary to these findings, increased availability of ACh can be protective in heart disease. For example, *in vivo* stimulation of the vagus nerve [13] or treatment with a cholinesterase inhibitor [14] led to improved outcome in experimental heart failure in rats. Recent data from Kanazawa *et al.* [15] have provided further evidence for cholinergic mediated cardioprotection. These authors found that cholinergic transdifferentiation of sympathetic neurons takes place during heart failure in rats, mice and humans. Moreover, inhibition of transdifferentiation in mice increased mortality in a mouse model of heart failure.

Multiple mechanisms may contribute to this protective effect of ACh on ventricular myocyte function. For example, increased parasympathetic tone can directly modulate heart function and also increase anti-inflammatory activity [16], which would be highly beneficial in heart failure. Furthermore, previous works have shown that ventricular cardiomyocytes express all the required components involved in ACh synthesis and release, and actively secrete ACh [17,18]. Yet, the relevance of this non-neuronal cardiomyocyte-derived cholinergic system in ventricular function is poorly understood. Here, we address the functional significance of this machinery in cardiac cells and show that cardiomyocyte released ACh can act in an autocrine/paracrine manner to mitigate the effects of adrenergic overstimulation. Together, our results point to another level of cholinergic control of cardiac function, which goes beyond the cardiac parasympathetic system, and is intrinsic to ventricular myocytes.

3.4 Materials and Methods

3.4.1 *Animal models*

In this study we used adult and neonatal C57BL/6 mice and Wistar rats. Rat and mouse neonatal cardiomyocytes were isolated from 2-4 and 2-day old animals, respectively. Adult ventricular myocytes were isolated from 10-12 week old male mice and from male Wistar rats weighing 220-250g. The VAChT knockdown homozygous mouse line (VAChT KD^{HOM} mice) [8] was previously described. Animals were maintained at UFMG, Brazil, and at the University of Western Ontario in accordance with NIH guidelines and the Canadian Council for Animal Care for the care and use of animals. Experiments were performed according to approved animal protocols from the Institutional Animal Care and Use Committee at UFMG (protocol# 016/11) and at the University of Western Ontario (2008-089).

3.4.2 *Neonatal cardiomyocyte culture*

Rat and mouse neonatal cardiomyocytes were cultured as previously described [19,20]. Briefly, cardiac cells were plated in dishes containing M199 medium supplemented with 100 units/ml penicillin, 100μg/ml streptomycin, 10% Fetal Bovine Serum and 2 mmol/L L-glutamine. To prevent growth of fibroblasts, medium was supplemented with 20 μg/mL cytosine-D-arabinofuranoside (ARA-c). After 48 hours, neonatal cardiomyocytes were exposed to isoproterenol (10 μmol/L) and/or pyridostigmine or neostigmine (10 μmol/L or 1 mmol/L) for 48 hours at the indicated concentrations. The cells were then used for immunofluorescence, Western blotting or qPCR analyses. When necessary, cells were incubated with atropine (10 μmol/L), vesamicol (5 μmol/L) or Nω-Nitro-L-Arginine Methyl Ester Hydrochloride (L-NAME, 10 μmol/L) for 48 hours. ACh (10 μmol/L) and phenylephrine (50 μmol/L) were also used in these experiments.

3.4.3 *Preparation of siRNA*

Potential target sites within the acetylcholinesterase gene were selected and then searched with NCBI Blast to confirm specificity for the enzyme. The siRNA for AChE was prepared as previously described by our group [21]. The sense and antisense oligonucleotides of siRNA were respectively, as follows: 5′-AAAAGGTGGTAGCATCCAATACCTGTCTC-3′ and 5′-AATATTGGATGCTACCACCTTCCTGTCTC-3′. In some studies we designed and tested a second siRNA targeting AChE (sense) 5′-AACGTATTGGTAGCAGACATTCCTGTCTC-3′ and (antisense) 5′-AAAATGTCTGCTACCAATACGCCTGTCTC-3′. For siRNA studies, neonatal cardiomyocyte cultures at day 4 were transfected with 100nM of siRNA. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 30 hours and then exposed to isoproterenol (10 μmol/L) for another 24 hours prior to use.

3.4.4 *NO measurement*

For these experiments we used freshly isolated adult ventricular myocytes from wild-type and VAChT KD^{HOM} mice aged 10-12 weeks. Measurement of NO production in living ventricular myocytes was done using the membrane permeable fluorescent indicator 4-amino-5-methylamino- 2′,7′-difluorofluorescein diacetate (DAF-FM diacetate, Invitrogen). To detect cytosolic NO, cardiomyocytes were loaded at 37°C with 5 μmol/ L DAF-FM diacetate for 30 min and then washed for 30 min with Tyrode solution. Cardiomyocytes were then incubated with ACh (10 μmol/L) or pyridostigmine (1 mmol/L) for 30 min. After this time, the fluorescence generated by NO production was recorded using a confocal microscope (CEMEL, UFMG). Analyses were performed with ImageJ software (NIH). In a separate group of experiments, cardiomyocytes were pre-incubated with atropine (10 μmol/L) or hemicholinium-3 (10 μmol/L) for 30 min before the addition of acetylcholine or pyridostigmine to the cells. In another set of experiments, we incubated mouse ventricular myocytes with different concentrations of ACh (0.1 to 10 μmol/L) or PYR (0.1 to 1000 μmol/L).

3.4.5 *FM1-43 FX*

Freshly isolated adult ventricular myocytes were loaded with FM1-43 FX dye (4 μM, Invitrogen) for 1 hour at 37°C in order to establish the localization of recycling vesicles. Before being fixed in a 4% paraformaldehyde (PFA) solution, the cells were ressuspended in Tyrode solution and washed for approximately 20 min to remove excess dye. Cells were then submitted to our immunofluorescence protocol.

3.4.6 *Statistical analysis*

All data are expressed as mean ± SEM, and the number of cells or experiments is shown as n. Significant differences between groups were determined with a Student's t-test or ANOVA followed by the Bonferroni post hoc test. Values of $p < 0.05$ were considered to be statistically significant.

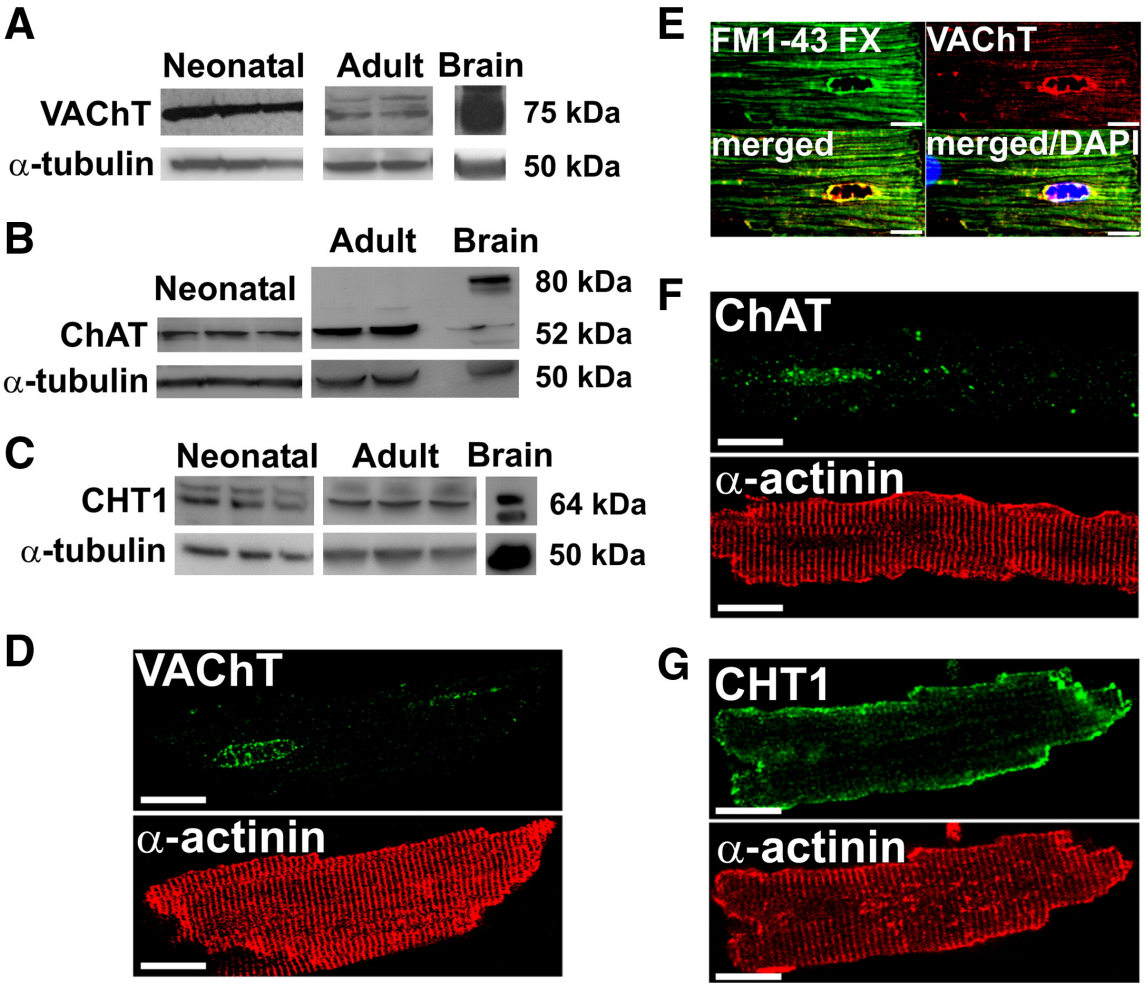
3.5 Results

3.5.1 *Cardiomyocytes present functional ACh synthesis and release machinery*

Previous work has shown that adult and neonatal rat cardiomyocytes express the three proteins involved in ACh synthesis and storage (ChAT, VAChT and CHT1) [17]. To further assess how ubiquitous this phenomenon is, we extended these findings and compared the expression of these three cholinergic markers in freshly isolated ventricular myocytes from adult and neonatal mice. As shown in Fig. 3.1A, both neonatal and adult cardiomyocytes express VAChT, observed as a 75 kDa band. For these experiments we used an anti-VAChT antibody that has previously been validated by using striatal specific VAChT knockout mice [23]. Our data show that the major band identified by this antibody is highly expressed in brain, and shows lower levels of expression in cardiomyocytes, as expected (Fig. 3.1A). ChAT, the enzyme responsible for ACh synthesis, was also detected in both neonatal cardiomyocytes and in adult ventricular cells (Fig. 3.1B). Interestingly, in cardiomyocytes, ChAT was observed mainly as a 52 kDa protein, in contrast with the more abundant 80 kDa protein found in the brain. The 52 kDa protein was also found in the brain, albeit in lower levels. It is possible that the 52 kDa protein is the result of alternative splicing of ChAT in cardiomyocytes. We also detected CHT1 in both adult and neonatal cardiomyocytes (Fig. 3.1C). We further analysed these cholinergic markers by using immunofluorescence and confocal microscopy to ascertain their localization in isolated adult mouse ventricular myocytes. Importantly, in cardiomyocytes, VAChT staining was found mainly in a perinuclear compartment, although VAChT punctate staining was also found in the cytoplasm (Fig. 3.1D). Because VAChT is a transmembrane protein usually located in endosomal, synaptic and recycling vesicles, we also tested whether this transporter would co-localize with the vital dye FM1-43 FX, a fixable analogue of FM1-43 which can be used to visualize recycling vesicles [24]. FM1-43 FX staining was observed all over the cardiac cell, but was enriched at the perinuclear region, where it extensively co-localized with VAChT (Fig. 3.1E). This finding is in agreement with previous data showing the

Figure 3.1 - Mouse cardiomyocytes express prototypical cholinergic markers. A–C. Representative Western blots of immunoreactivity for VAChT, ChAT, and CHT1 in neonatal and adult mouse ventricular myocytes. Brain samples were used as a positive control. α-tubulin was used as a loading control. **D–G.** VAChT, FM1-43 FX, ChAT and CHT1 staining in adult mouse ventricular myocytes. Cardiomyocytes were also labeled with antibody against the sarcomeric protein α-actinin to show cellular organization. Scale bar = 10 μm.

Figure 3.1 - Mouse cardiomyocytes express prototypical cholinergic markers.

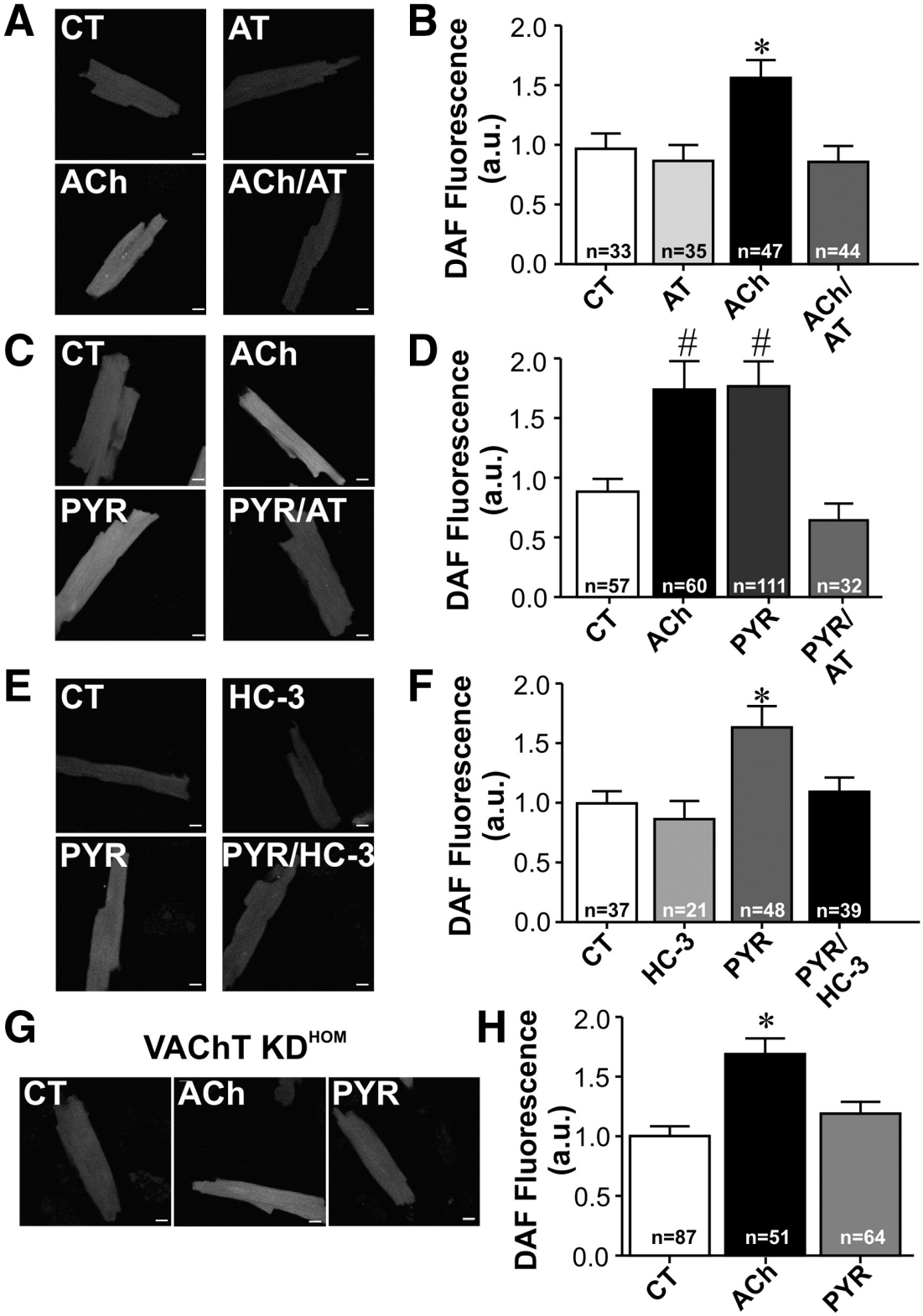


presence of vesicle-associated membrane proteins (VAMP) in a punctate perinuclear pattern in cardiomyocytes [25]. In addition, we observed ChAT immunostaining in the nuclear region in close proximity to where VAChT is localized (Fig. 3.1F). The nuclear localization of ChAT matches previous findings in neurons [26]. In agreement with the Western blot data, CHT1 was also found in isolated cardiomyocytes. However, in contrast with the localization of VAChT and ChAT, CHT1 was mostly localized to the plasma membrane (Fig. 3.1G), where it presumably plays an active role in the reuptake of choline.

A critical issue in identifying ACh secretion in non-neuronal cell culture is its rapid degradation by AChE, an enzyme that has very high catalytic activity and is abundantly expressed in cardiomyocytes [17]. Furthermore, detection of very low levels of ACh is usually a challenge. One of the many responses of cardiomyocytes to ACh is an increase in NO levels [27]. Therefore, we next sought to determine whether we could use the generation of NO as a biological sensor for the effects of cardiomyocyte-derived ACh. Thus, freshly isolated ventricular myocytes from adult wild-type mice were loaded with the fluorescent indicator DAF-FM to assess NO generation in response to ACh (Fig. 3.2A-F). Fig. 3.2A shows representative images of DAF fluorescence in mouse ventricular myocytes. Average values (Fig. 3.2B) show that exogenously added ACh (10 μmol/L) induced a significant increase in NO generation in ventricular myocytes, which was blocked by atropine, a muscarinic antagonist. As shown in Fig. 3.2B, atropine alone had no effect on NO generation. In order to test if ACh released by cardiomyocytes could act in these cells in an autocrine/paracrine manner, we used a reversible cholinesterase inhibitor (pyridostigmine) as a way to increase the availability of cardiomyocyte derived ACh, by preventing its degradation by AChE. Wild-type cardiomyocytes were incubated with pyridostigmine (1 mmol/L; 30 minutes), and DAF fluorescence was assessed. As shown in Fig. 3.2C-D, adult mouse ventricular myocytes treated with pyridostigmine alone presented increased NO generation to levels similar to that found in cells exposed to exogenous ACh. This effect was completely blocked by atropine. To investigate if the effect of pyridostigmine on NO generation was dependent on CHT1 activity, we treated

Figure 3.2 - NO levels can be used as a biosensor to detect ACh release in cardiomyocytes. A–F. DAF fluorescence measurements performed in wild-type (WT) cardiomyocytes. **A.** Sample confocal images show cellular increase in DAF fluorescence in cardiomyocytes from WT mice treated with ACh. **B.** Averaged DAF fluorescence increase in adult ventricular myocytes following acute treatment with exogenously added ACh for 30 min. The pre-incubation of muscarinic receptor antagonist, atropine (AT), inhibits the increase in DAF fluorescence in ACh treated cardiomyocytes. **C.** Sample images of DAF fluorescence of cardiomyocytes incubated with ACh, pyridostigmine (PYR) or PYR/AT. **D.** Effect of cholinesterase inhibition on DAF fluorescence in the presence or absence of atropine (AT) pre-treatment. Cardiomyocytes exposed to pyridostigmine presented an increase in DAF fluorescence, which was blunted by atropine. **E.** Sample images of DAF loaded cardiac myocytes treated with hemicholinium-3 (HC-3) and/or PYR for 30 min. **F.** HC-3 blunted PYR induced increase in DAF fluorescence in ventricular myocytes. **G.** Sample images of DAF fluorescence in ventricular myocytes of VAChT KD^{HOM} mice incubated with ACh or PYR. **H.** Cholinesterase inhibition in cardiomyocytes with reduced VAChT expression levels does not lead to a significant increase in NO generation. n = number of cells analyzed. *$p < 0.05$ when compared to the other groups. #$p < 0.05$ when compared to CT and PYR/AT groups. All cells were loaded with fluorescent dye following the same protocol, and imaging was done preserving the same parameters in both control and drug treated cardiomyocytes. Scale bar = 10 μm.

Figure 3.2 - NO levels can be used as a biosensor to detect ACh release in cardiomyocytes.



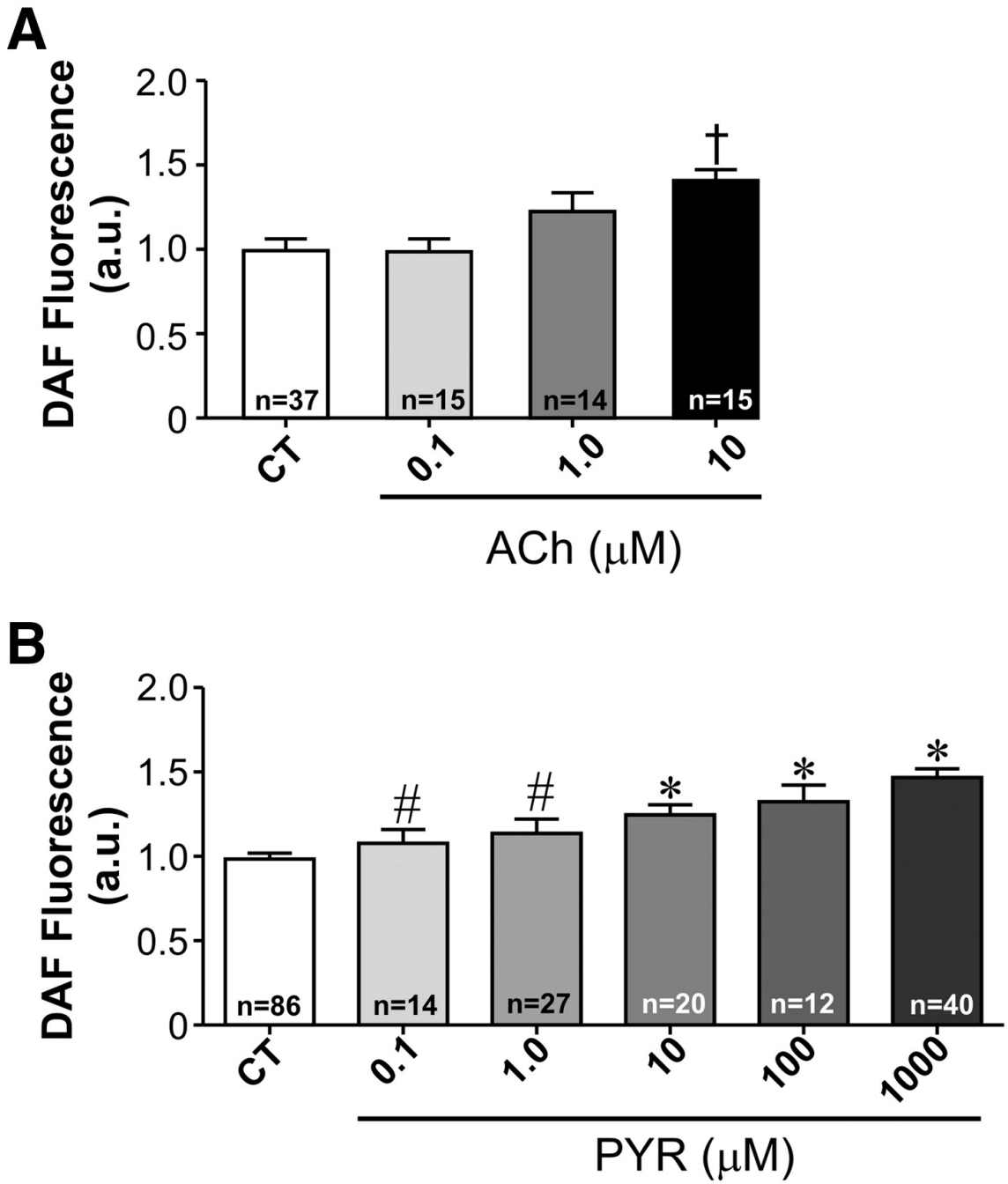
the cells with hemicholinium-3 (HC-3, 10 μmol/L), a CHT1 inhibitor that abolishes ACh synthesis. Fig. 3.2E-F shows that pre-incubation of cardiomyocytes with HC-3 also prevented the effects of pyridostigmine on DAF fluorescence. Similar to atropine, HC-3 alone had no effect on DAF fluorescence.

To assess the specificity of pyridostigmine effect on NO production we compared the concentration dependency of ACh and pyridostigmine on DAF loaded ventricular myocytes. Cardiac cells were exposed to increased concentrations of ACh and pyridostigmine and DAF fluorescence was assessed. As shown in Fig. 3.3A-B, both ACh and pyridostigmine produced a concentration dependent increase in NO levels, thus eliminating the possibility that pyridostigmine exerts off target effects.

We then examined the effects of pyridostigmine on DAF fluorescence in cardiomyocytes from VAChT KD^{HOM} mice, an animal model that presents 70% reduction in VAChT levels and concomitant decrease in ACh release [8]. Fig. 3.2G-H shows that cardiomyocytes obtained from VAChT KD^{HOM} mice fail to respond to pyridostigmine. This finding contrasted with the significant response to pyridostigmine found in wild-type myocytes (Fig. 3.2C-F). Moreover, exogenously added ACh elicited a significant increase in DAF fluorescence in cardiomyocytes from VAChT KD^{HOM} mice. The response to ACh found in these cells was similar to the one observed in wild-type cardiomyocytes (Fig. 3.2B,D). Therefore, by using a cholinesterase inhibitor, it is possible to increase the availability of cardiomyocyte derived ACh, which in turn activates muscarinic receptors to increase NO levels. Taken together, these data show that cardiomyocytes are capable of synthesizing and releasing ACh confirming the functionality of cholinergic machinery in these cells. Moreover, it seems that both choline reuptake and ACh storage in VAChT positive organelles are required for ACh secretion from adult cardiomyocytes.

Figure 3.3 - Concentration-dependent effects of ACh and PYR on NO generation in mouse ventricular myocytes. A-B. Averaged DAF fluorescence increase in adult ventricular myocytes following acute treatment with exogenously added ACh for 20 minutes. Bar graph shows a progressive increase in cardiomyocyte induced NO production upon ACh (0.1 to 10 μmol/L) or pyridostigmine (PYR) stimulation. PYR doses ranged from 0.1 to 1000 μmol/L. n= number of cardiomyocytes analysed. † $p<0.05$ when compared to control and ACh 0.1 μmol/L. * $p<0.05$ when compared to control. # $p<0.05$ when compared to PYR 1000 μmol/L.

Figure 3.3 - Concentration-dependent effects of ACh and PYR on NO generation in mouse ventricular myocytes.



3.5.2 *Cholinesterase inhibition prevents adrenergic hypertrophic signaling*

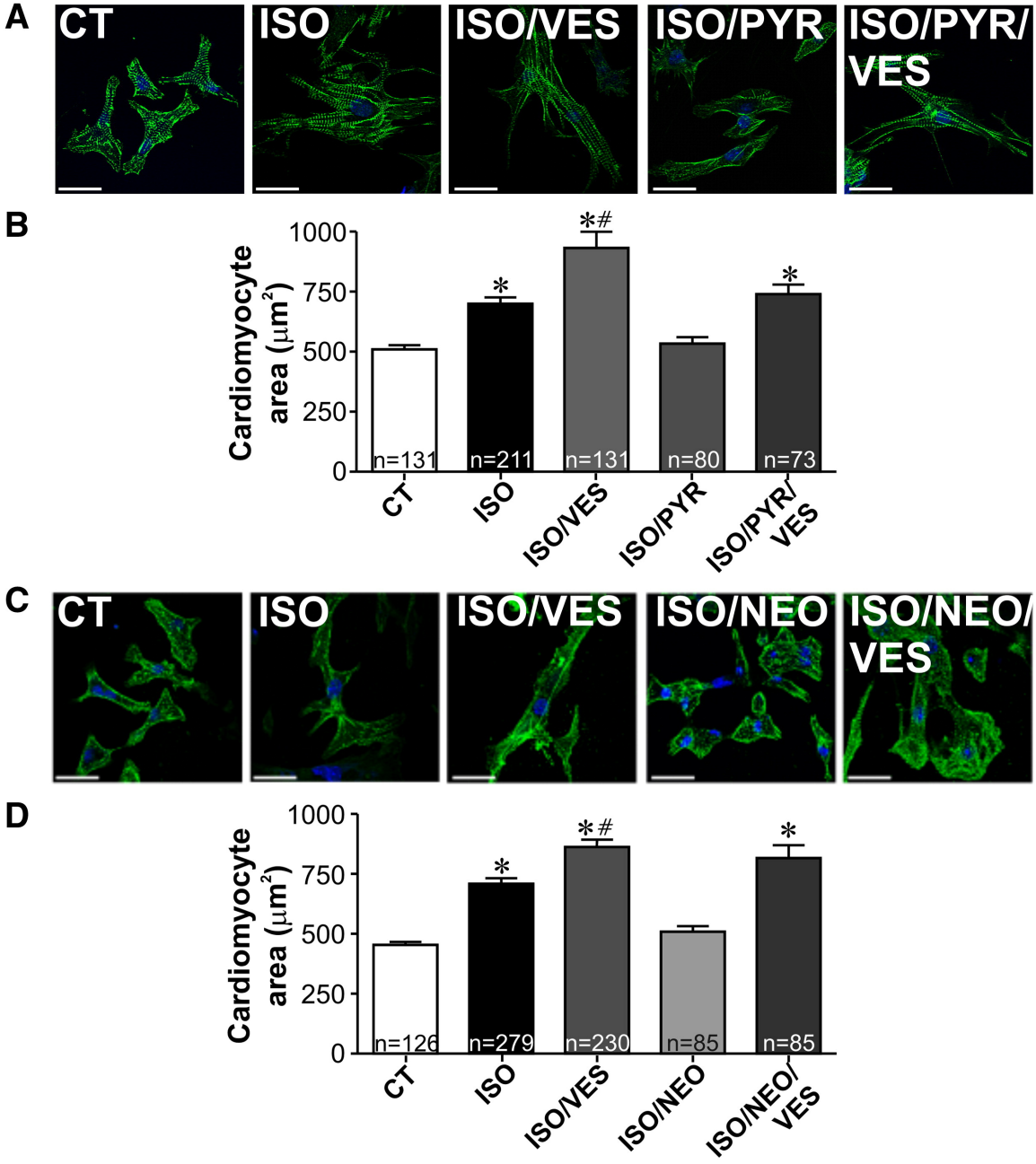
Our data indicate that cholinesterase inhibition increases the availability of ACh secreted by cardiomyocytes. Therefore, we investigated the possibility that this cardiomyocyte cholinergic signaling can exert protective effects. Thus, the next group of experiments was conducted in mouse neonatal cardiomyocytes as these cells are easier to maintain in culture than isolated adult cells and their phenotype is highly stable [28,29]. In order to simulate disease conditions, neonatal cardiomyocytes were treated with isoproterenol (ISO, 10 μmol/L) for 48 hours and cellular hypertrophy was assessed by direct measurement of myocyte surface area. Fig. 3.4A shows representative immunofluorescence images from α-actinin stained cardiomyocytes. Cardiomyocytes treated with isoproterenol demonstrated increased cell surface area by 40% (Fig. 3.4B), whereas concomitant treatment with vesamicol (5 μmol/L), a specific VAChT inhibitor, caused an even greater increase in cell surface area (80%), thus implicating released ACh in the endogenous protection against cellular hypertrophy. Importantly, pyridostigmine (10 μmol/L) prevented the hypertrophic effect of isoproterenol. Similar effects were observed in cells treated with 1 mmol/L pyridostigmine (data not shown). The action of pyridostigmine was dependent on VAChT activity, and ACh secretion, as isoproterenol was again able to effectively induce hypertrophy when vesamicol was present in the media. We further confirmed these data in a second cohort of cells that were treated with isoproterenol and another cholinesterase inhibitor, neostigmine (10 μmol/L). Fig. 3.4C-D shows that neostigmine was as effective as pyridostigmine in preventing the hypertrophic actions of isoproterenol. We again found that vesamicol was able to block the protective effect of neostigmine. Together, these data suggest that ACh released by cardiomyocytes can offset hypertrophic effects of isoproterenol on mouse neonatal cardiac cells.

3.5.3 *Mechanisms involved in mitigation of isoproterenol-induced hypertrophy*

In order to further understand the mechanisms involved in this protective response to endogenously secreted ACh, we used rat cardiomyocytes, which also have an intrinsic

Figure 3.4 - Cholinesterase inhibition in mouse neonatal cardiomyocytes prevents isoproterenol induced hypertrophy. A. Sample immunofluorescence images of α-actinin/DAPI stained neonatal cardiac cells. Isoproterenol (ISO) induced a significant increase in cardiomyocyte surface area, an effect that was more pronounced in the presence of vesamicol (VES), and abolished by pyridostigmine (PYR). Pyridostigmine effects on isoproterenol treated cells were eliminated by vesamicol. **B.** Bar graph of cardiomyocyte surface area measurements. *$p<0.05$ when compared to control and ISO/PYR. #$p<0.05$ when compared to ISO and ISO/PYR/VES. **C-D.** Isoproterenol induced hypertrophy was blunted by neostigmine (NEO). Neostigmine effects were prevented in the presence of vesamicol. *$p<0.05$ when compared to control and ISO/NEO. # $p<0.05$ when compared to ISO. n= number of cells analysed in each experimental group. Scale Bar=25μm.

Figure 3.4 - Cholinesterase inhibition in mouse neonatal cardiomyocytes prevents isoproterenol induced hypertrophy.



cholinergic system, as previously demonstrated by others [17,18], and are easier to culture than mouse cells [30]. Initially, we assessed expression levels of cholinergic markers in rat cardiomyocytes (Fig. 3.5). Overall these data match previous findings in the literature [17,18], and also confirm our data obtained from mouse cardiomyocytes, as we show that rat cardiomyocytes express VAChT, ChAT, M_2-AChR and AChE. In adult rat ventricular cells, VAChT is detected as a 75 kDa and a 47 kDa band (Fig. 3.5A). VAChT expression was also observed in neonatal rat cardiomyocytes isolated from 2-4 day old rats, although in these cells VAChT was observed at an intermediate size band, as previously reported [17]. These differences in molecular mass are probably due to different glycosylation patterns of VAChT molecules [31,32]. ChAT was also detected in both cellular systems (Fig. 3.5B). In addition, we show that adult and neonatal cardiomyocytes express AChE (Fig. 3.5C), as shown by others [33].

Our finding that VAChT is presented at the perinuclear region is potentially important since it suggests that cardiomyocytes segregate the exocytotic machinery at the nuclear region, as observed for other granules in these cells [25,34]. In order to confirm this finding, we stained isolated rat adult ventricular myocytes and imaged them by confocal microscopy. As previously observed, VAChT is mainly found at the perinuclear region where it colocalized with recycling vesicles stained with FM1-43 FX (Fig. 3.5D, arrow). Some sparse VAChT positive vesicles were also observed along the T-tubular system (Fig. 3.5D, arrowhead). In addition, we observed ChAT immunostaining in the nuclear and intercalated disk regions, as well as in T-tubules, with some presence in the perinuclear area (Fig. 3.5E). M_2-AChR receptor immunostaining was also enriched at the perinuclear region, with some staining found at the T-tubules (Fig. 3.5F). Corroborating the data obtained from adult ventricular myocytes, neonatal rat cardiomyocytes also showed intense staining and similar subcellular localization for both VAChT (Fig. 3.5G) and ChAT (Fig. 3.5H). Taken together, our data from mice and rats confirm the presence of prototypical cholinergic markers in adult and neonatal cardiomyocytes and show for the first time that VAChT positive vesicles segregate to the nuclear periphery along with other cholinergic markers.

Figure 3.5 - Rat cardiomyocytes express neuronal cholinergic markers. A-C. Western blots of VAChT, ChAT and AChE in neonatal and adult rat ventricular myocytes. **D.** VAChT distribution in adult ventricular myocytes. FM1-43 FX-loaded vesicles exhibited a punctuate fluorescence mainly at the perinuclear region of ventricular myocytes, which colocalizes with VAChT. VAChT vesicles at perinuclear hotspots are indicated by the yellow arrow. Some sparse VAChT staining was also found at T-tubules (arrowhead). **E.** ChAT immunolocalization was found at the nuclear region, T-tubular system and intercalated disks. **F.** M_2-AChR staining is localized to the perinuclear area and T-tubular system. **G-H.** VAChT and ChAT staining are also detected in neonatal rat cardiomyocytes. Similar staining patterns were observed between neonatal and adult cardiomyocytes. Arrows show VAChT presence at the perinuclear and cytosolic regions. Cardiomyocytes were also labeled with an antibody against the sarcomeric protein α-actinin to show cellular organization. Scale Bar=10μm.

Figure 3.5 - Rat cardiomyocytes express neuronal cholinergic markers.

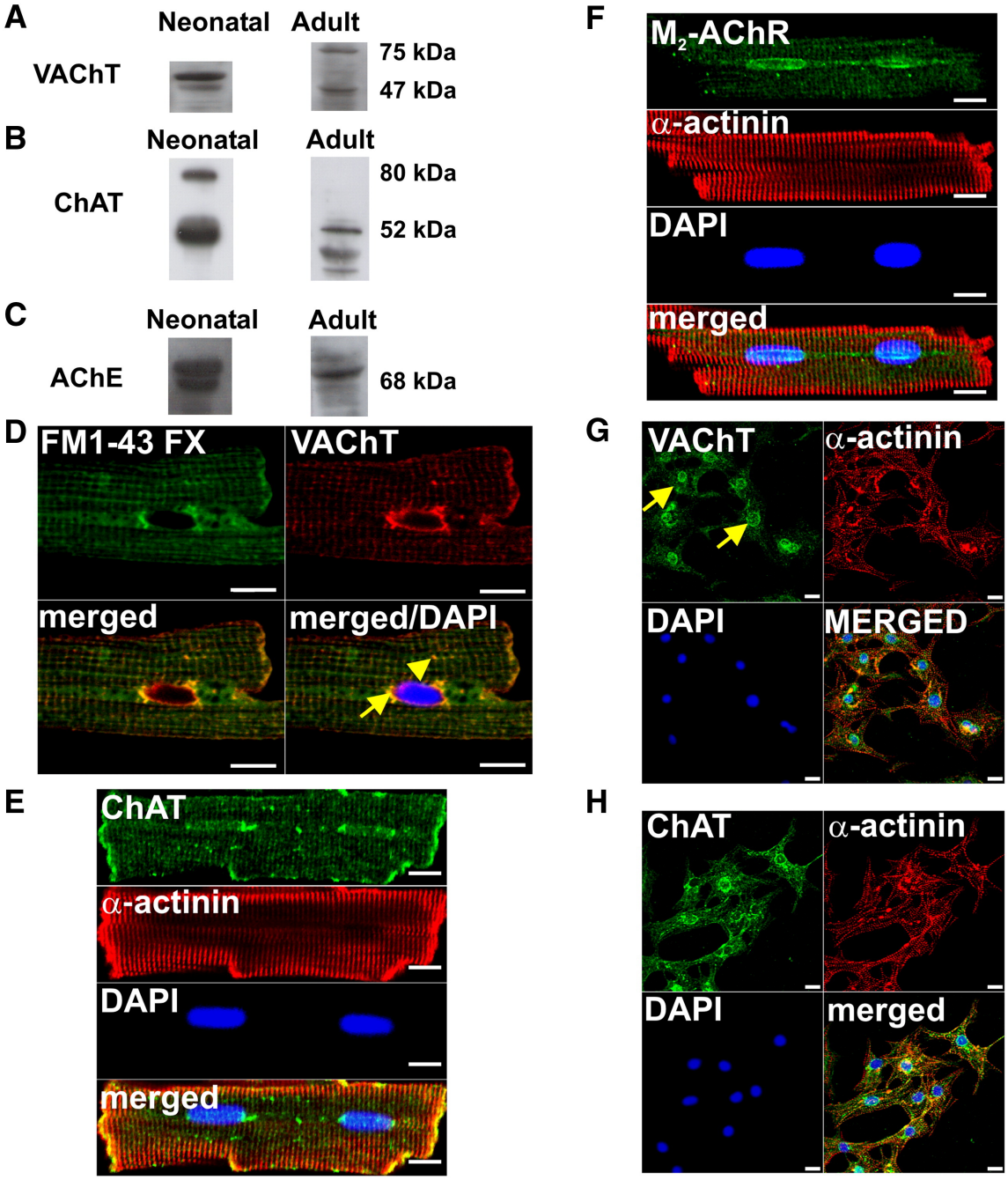
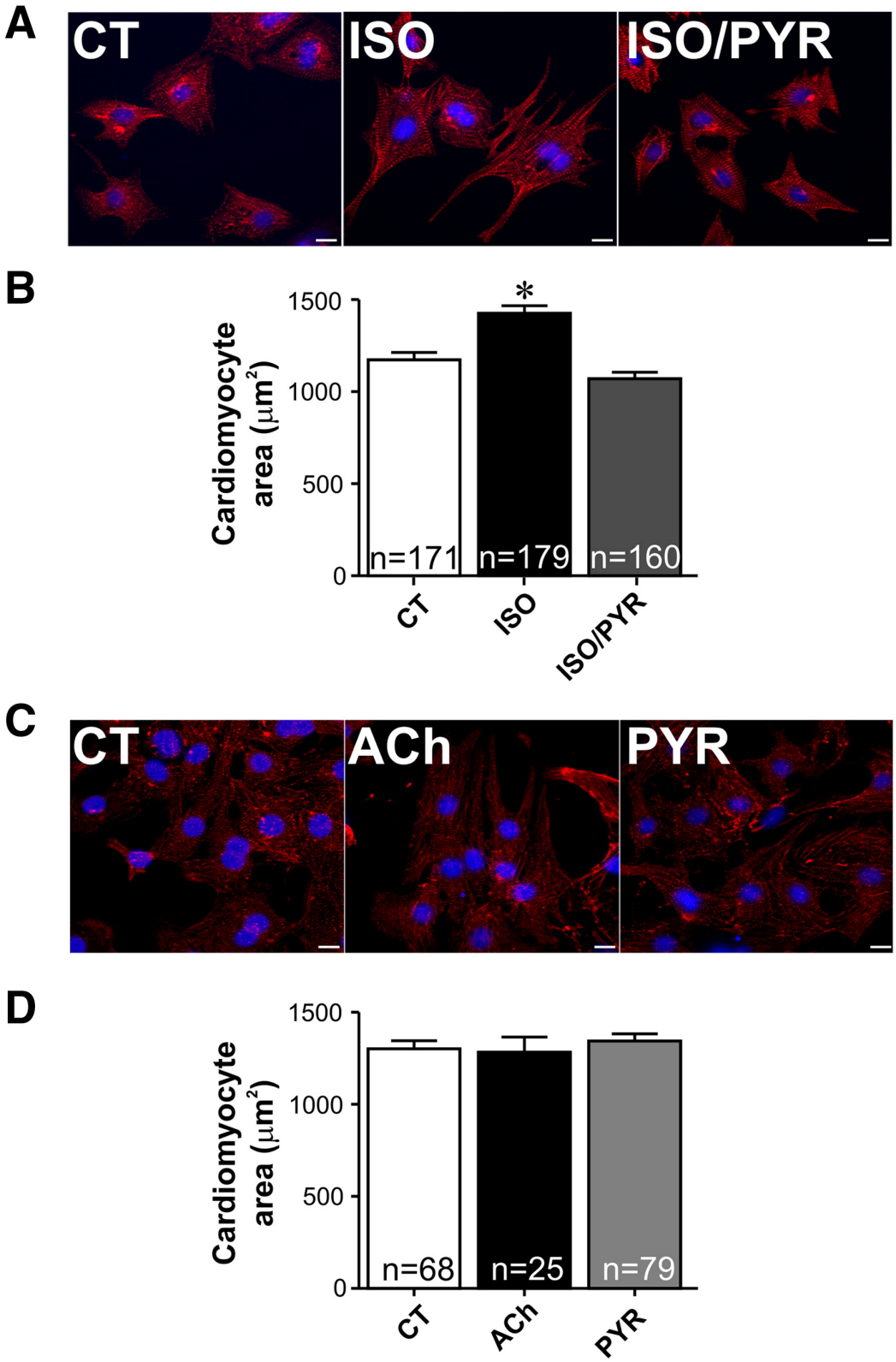


Figure 3.6 - Cholinesterase inhibition suppresses hypertrophy induced by isoproterenol in rat neonatal cardiomyocytes. A. Representative immunofluorescence images from α-actinin/DAPI stained neonatal cardiomyocytes in control (CT), isoproterenol (ISO) and isoproterenol/pyridostigmine (ISO/PYR) treated groups. **B.** Quantification of cardiomyocyte surface area from experiments shown in **A**. **C–D.** Representative immunofluorescence images and quantification of cellular surface area from α-actinin/DAPI stained neonatal cardiomyocytes from CT, ACh and PYR treated groups. *$p < 0.05$ when compared to control and ISO/PYR groups. n = number of cells analyzed in each group. Scale bar = 10 μm.

Figure 3.6 - Cholinesterase inhibition suppresses hypertrophy induced by isoproterenol in rat neonatal cardiomyocytes.



In order to gain mechanistic insight into the functionality of this system, and to verify whether its anti-hypertrophic action is conserved between mice and rats, we exposed neonatal rat cardiomyocytes to isoproterenol and pyridostigmine. As shown in Fig. 3.6, pyridostigmine was again effective in preventing the hypertrophic effects of isoproterenol. Importantly, neither pyridostigmine nor ACh (10 μmol/L) alone altered the cell surface area of neonatal rat cardiomyocytes (Fig. 3.6C-D). To further confirm these findings, we depleted endogenous acetylcholinesterase in rat neonatal cardiomyocytes using specific siRNA targeting AChE. Real-time PCR shows the efficiency of siRNA induced reduction of AChE mRNA (by approximately 80%, Fig. 3.7A). Treatment of cardiomyocytes with isoproterenol is well known to increase expression levels of atrial natriuretic peptide (ANP) [35] and β-myosin heavy chain (β-MHC) transcripts [36]. Therefore, the expression levels of these transcripts was analysed by qPCR. As expected, we observed a significant upregulation of ANP and β-MHC levels upon isoproterenol stimulation of neonatal rat cardiomyocytes. Importantly, AChE silencing partially abolished the effect of isoproterenol on ANP and β-MHC expression (Fig. 3.7B-C). Similar findings were observed with a distinct siRNA targeted to AChE (data not shown). Thus these data further support the hypothesis that preservation of endogenously secreted ACh prevents the activation of the hypertrophic signaling cascade induced by isoproterenol in cardiomyocytes.

Similarly, the effect of isoproterenol on β-MHC mRNA was also abolished in cardiomyocytes treated with the pharmacological cholinesterase inhibitor, pyridostigmine (Fig. 3.8A). In order to gain mechanistic insight into how cardiomyocyte derived ACh exerts its anti-hypertrophic effects, we investigated whether nitric oxide was involved in this process. Thus, we assessed the effect of L-NAME on β-MHC mRNA levels in neonatal rat cardiomyocytes treated with isoproterenol and pyridostigmine. Inhibition of nitric oxide production with L-NAME significantly blunted pyridostigmine induced β-MHC transcript downregulation in isoproterenol treated cardiomyocytes, indicating a role for NO in the anti-hypertrophic response elicited by intrinsic ACh (Fig. 3.8A). Moreover, isoproterenol induced ANP upregulation was also abolished in neonatal cardiomyocytes incubated with pyridostigmine (Fig. 3.8B), supporting our findings obtained with AChE-

Figure 3.7 - AChE silencing attenuates isoproterenol induced fetal gene expression in neonatal cardiomyocytes. A. siRNA targeting AChE significantly reduces mRNA expression levels of this enzyme. **B–C.** Isoproterenol treatment significantly increases ANP and β-MHC mRNA levels in neonatal cardiomyocytes, an effect that is partially abolished in cells transfected with siRNA targeting AChE. n = 4 to 8 samples from each group. *$p < 0.05$ when compared to the other groups.

Figure 3.7 - AChE silencing attenuates isoproterenol induced fetal gene expression in neonatal cardiomyocytes.

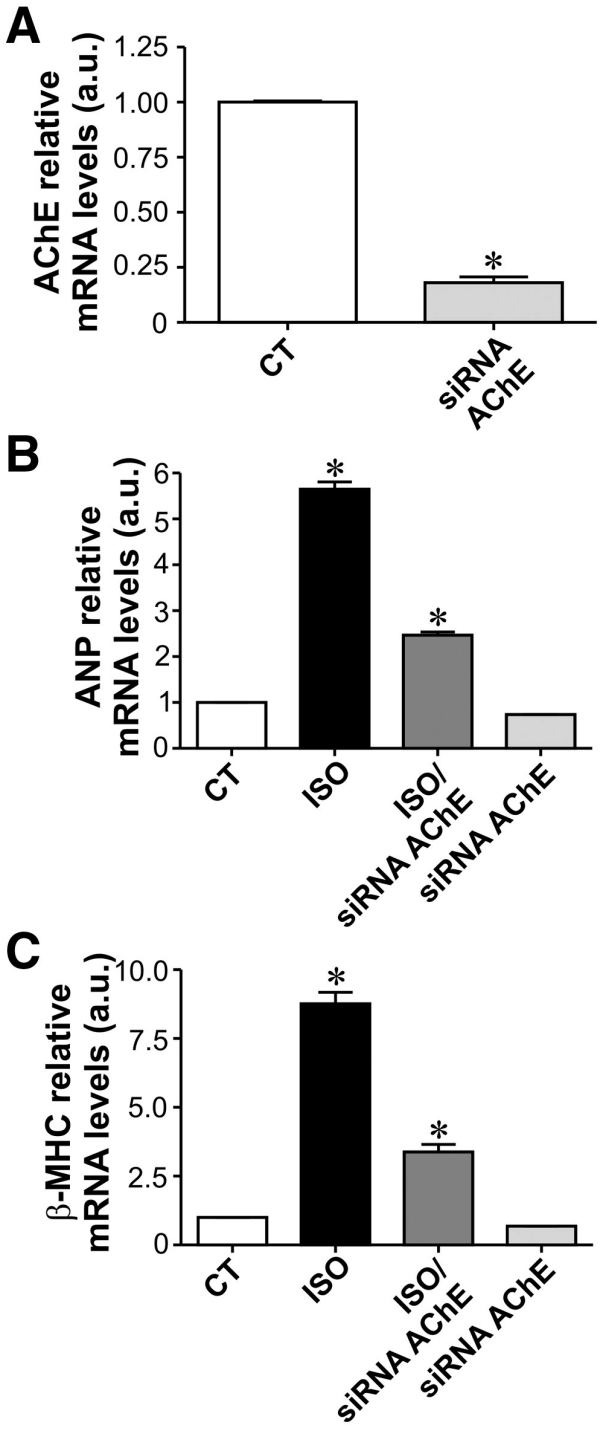
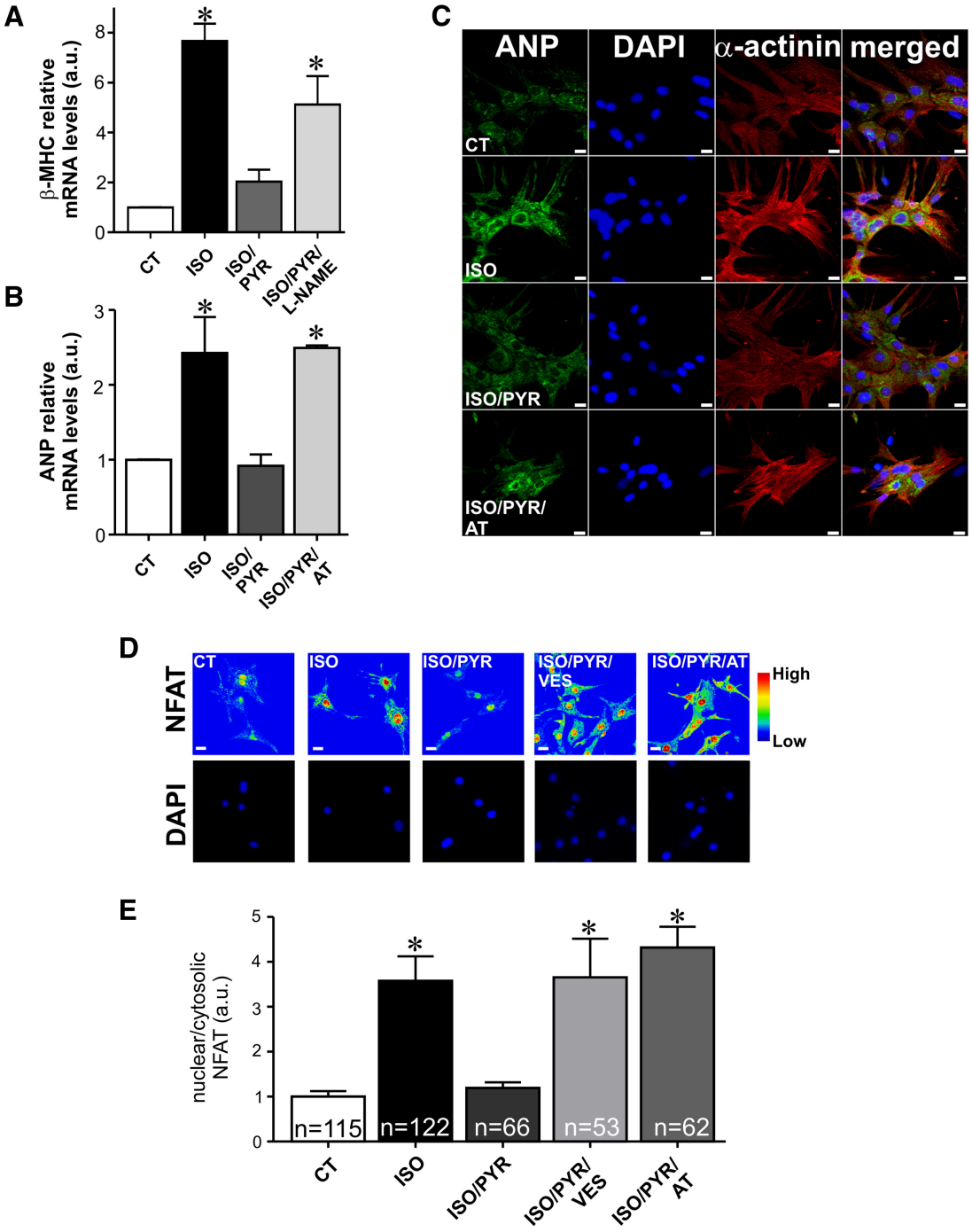


Figure 3.8 - Cholinesterase inhibition prevents isoproterenol induced remodeling in neonatal rat cardiomyocytes. A. Cholinesterase inhibition in neonatal cardiomyocytes prevents isoproterenol (ISO) induced upregulation of β-MHC mRNA, an effect that is lost in the presence of L-NAME. $n = 4$ to 5 samples from each group. **B.** qPCR experiments revealed a significant upregulation in ANP transcript levels in ISO treated cells. Pyridostigmine prevented ANP upregulation induced by ISO treatment. The effect of ISO on ANP transcription in pyridostigmine (PYR) treated cells was restored upon exposure to atropine (AT). $n = 3$ to 5 samples from each group. **C.** ISO stimulation increased perinuclear ANP staining with levels of staining in ISO/PYR treated cardiomyocytes comparable to that in control cells. The ability of PYR to prevent ANP increase under ISO stimulation was blunted by atropine. **D.** In control cells, NFAT is found mainly in the cytosol, and upon ISO stimulation this pro-hypertrophic transcription factor translocates to the nucleus. ISO induced NFAT translocation was completely suppressed by PYR. Addition of either vesamicol or atropine significantly inhibited PYR effects on NFAT localization in ISO treated cells. **E.** Bar graph showing the relative amount of nuclear/cytosolic NFAT. n = number of cells analyzed. $*p < 0.05$ when compared to control and ISO/PYR groups. Scale bar = 10 μm.

Figure 3.8 - Cholinesterase inhibition prevents isoproterenol induced remodeling in neonatal rat cardiomyocytes.



siRNA. Interestingly, the effect of pyridostigmine in preventing ANP transcript upregulation was inhibited by atropine (Fig. 3.8B), confirming the finding that available ACh after pyridostigmine treatment uses muscarinic receptors. ANP protein localization was also analysed in the presence of isoproterenol by immunostaining (Fig. 3.8C). Isoproterenol stimulation resulted in significantly increased perinuclear ANP staining, an effect that was prevented by pyridostigmine. Similar results were observed when isoproterenol treated cells were exposed to neostigmine (Fig. 3.9). The ability of pyridostigmine to prevent ANP increase under isoproterenol stimulation was lost when cardiomyocytes were exposed to atropine (Fig. 3.8C).

A prominent signaling cascade stimulated by isoproterenol, and whose overactivation is associated with hypertrophic remodeling in cardiomyocytes, is the nuclear factor of activated T cells (NFAT) pathway. In neonatal cardiomyocytes, isoproterenol treatment leads to NFAT translocation to the nucleus, as previously described by others [37]. Fig. 3.8D-E shows the localization of NFAT in non-treated neonatal rat cardiomyocytes and demonstrates that isoproterenol stimulation leads to increased immunoreactivity for NFAT in the nucleus. The isoproterenol induced nuclear translocation of NFAT was completely prevented by pyridostigmine. Similar finding was observed in isoproterenol treated neonatal cardiomyocytes that were exposed to ACh (Fig. 3.10). Addition of vesamicol, a specific inhibitor of the VAChT, or atropine, significantly blunted the effects of pyridostigmine on NFAT translocation in isoproterenol treated cells (Fig. 3.8D-E).

To further assess the possible mechanism through which ACh released by cardiomyocytes prevents isoproterenol-induced hypertrophy, we examined Ca^{2+} transients in isolated adult rat cardiomyocytes kept in culture for 20 hours. Activation of β-adrenergic receptors by isoproterenol in ventricular myocytes is known to enhance the magnitude of the intracellular Ca^{2+} transient, $[Ca^{2+}]_i$, which contributes to the alterations in intracellular signaling. It is also known that ACh binds to M_2-AChR which couple to $G_{i/0}$ family of G proteins [38] and inhibits adenylyl cyclase. In many ways, therefore, the effects of ACh are opposed to those of sympathetic activation. Freshly

Figure 3.9 - Neostigmine exerts anti-hypertrophic effects. Representative images of neonatal cardiomyocytes stained with anti-ANP (green) are displayed. The nucleus was stained with DAPI (blue). Neostigmine (NEO) treatment abolished isoproterenol (ISO) induced changes in ANP localization. Scale Bar= 10μm.

Figure 3.9 - Neostigmine exerts anti-hypertrophic effects.

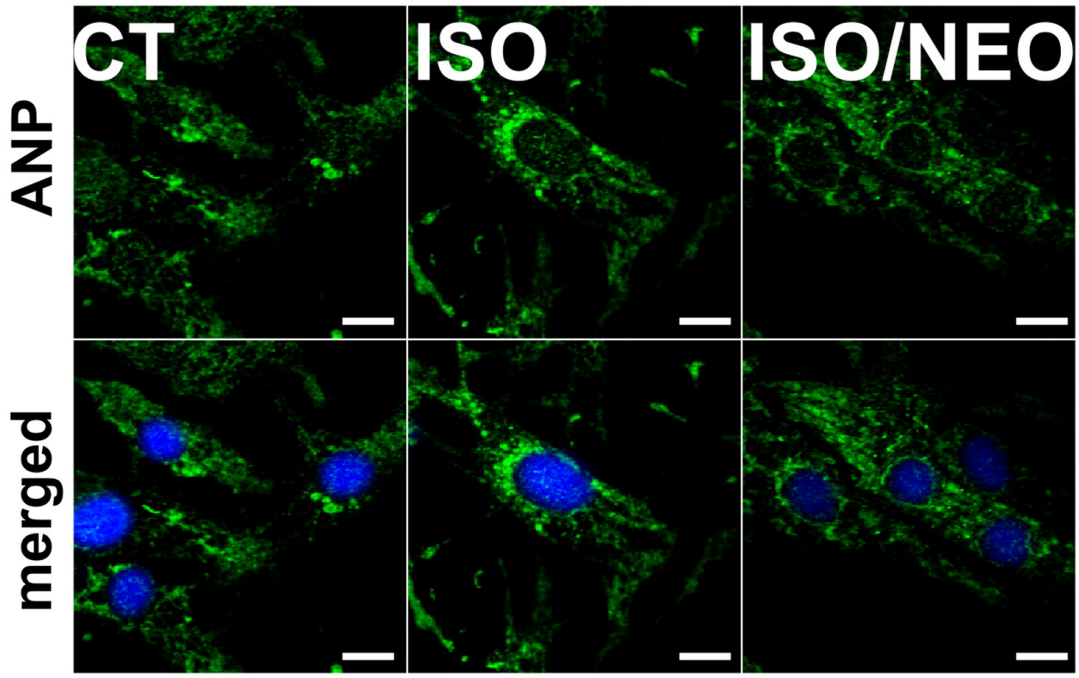


Figure 3.10 - ACh blunts isoproterenol induced NFAT translocation. Neonatal rat cardiomyocytes were labeled with antibody against NFAT (green). The nucleus was stained with DAPI (blue). ACh blunts hypertrophic signaling induced by isoproterenol by inhibiting NFAT nuclear translocation in rat neonatal cardiomyocytes. Scale Bar=10μm.

Figure 3.10 - ACh blunts isoproterenol induced NFAT translocation.

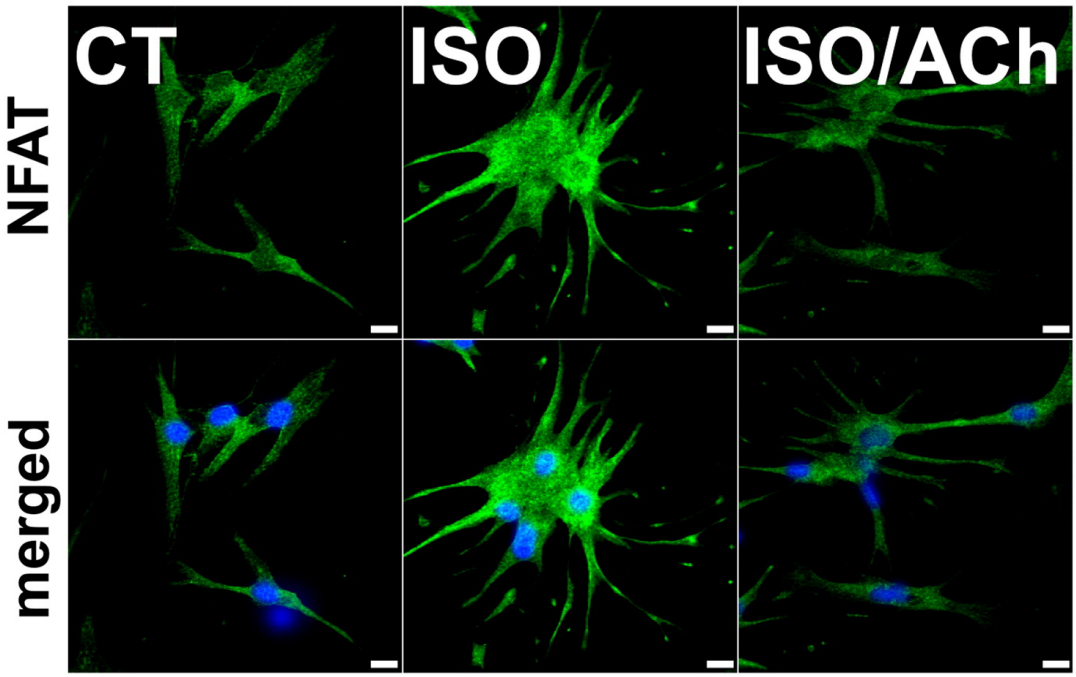
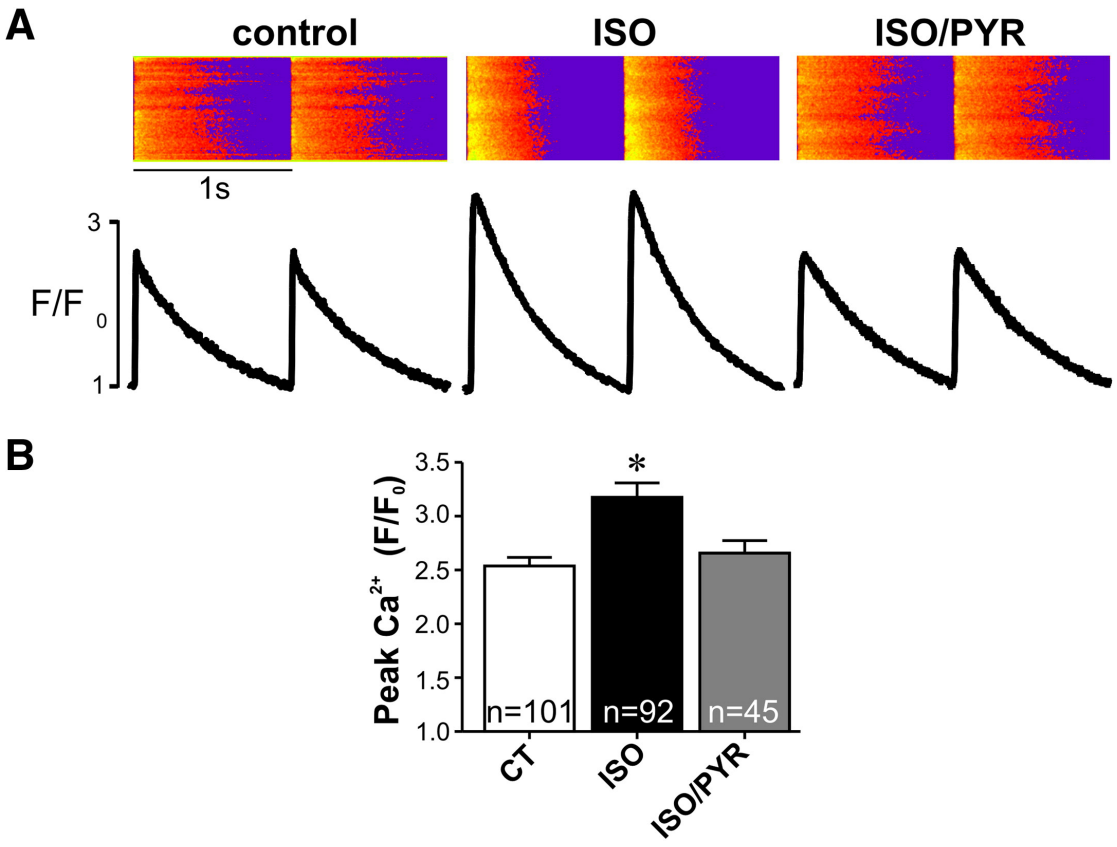


Figure 3.11 - Cardiomyocyte cholinergic signaling prevents isoproterenol effects on Ca^{2+} transients. A. (Top) Sample Ca^{2+} transient images recorded in Fluo-4/AM loaded-ventricular myocytes kept in culture for 20 h. **(Bottom)** Ca^{2+} transient profile. **B.** Averaged bar graph showing the increase in Ca^{2+} transient following isoproterenol stimulation. Cholinesterase inhibition by pyridostigmine prevented isoproterenol effects on Ca^{2+} transient amplitude. n = number of cells analyzed. * = $p < 0.05$ when compared to the other groups.

Figure 3.11 - Cardiomyocyte cholinergic signaling prevents isoproterenol effects on Ca^{2+} transients.



isolated rat ventricular myocytes were plated in laminin-coated dishes and treated with isoproterenol (1 μmol/L). After a 20 hour treatment, cells were loaded with Fluo-4/AM for 30 min, and Ca^{2+} transient parameters were examined in a confocal microscope. (Fig. 3.11A) displays typical line scan fluorescence images recorded from electrically stimulated ventricular myocytes. Chronic activation of β-adrenergic receptor by isoproterenol increases Ca^{2+} transient amplitude, and this, in part, is responsible for the adrenergic regulation of myocytes (Fig. 3.11B). Concomitant addition of pyridostigmine (500 μmol/L) to isoproterenol-treated myocytes significantly attenuated the increase in Ca^{2+} transient amplitude induced by isoproterenol thus suggesting an opposing effect of pyridostigmine. Collectively, these data support the functionality of ACh synthesis and release machinery in ventricular myocytes, and indicate that non-neuronal ACh can activate muscarinic receptors to counteract the effect of isoproterenol at the calcium signaling level.

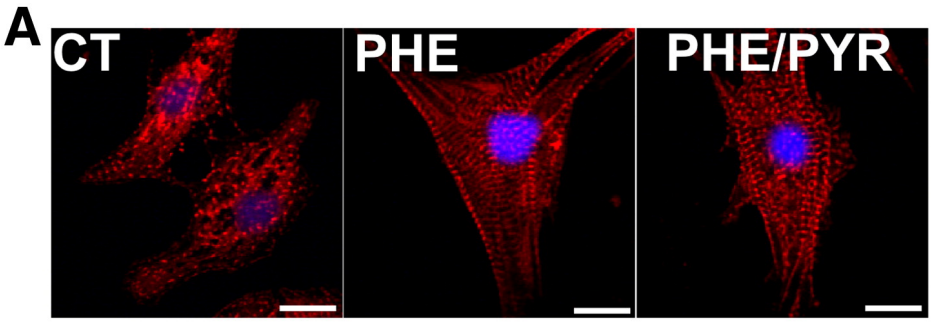
We next investigated whether pyridostigmine could also inhibit the hypertrophic response induced by another adrenergic stimulus, phenylephrine, which is known to activate a Gq signaling pathway through α-adrenergic receptors stimulation [39]. Neonatal rat cardiomyocytes treated with 50 μmol/L phenylephrine for 48 hours presented a 39% increase in cell surface area when compared to control cardiomyocytes (Fig. 3.12A-B). This hypertrophic effect was partially prevented by pyridostigmine. Moreover, pyridostigmine significantly attenuated phenylephrine-induced upregulation of ANP transcript levels (Fig. 3.12C). Once again, atropine and L-NAME were effective in preventing pyridostigmine effects on phenylephrine treated neonatal cardiomyocytes. Collectively, our data show that increased availability of released ACh prevents phenylephrine-induced pathological remodeling in cardiomyocytes.

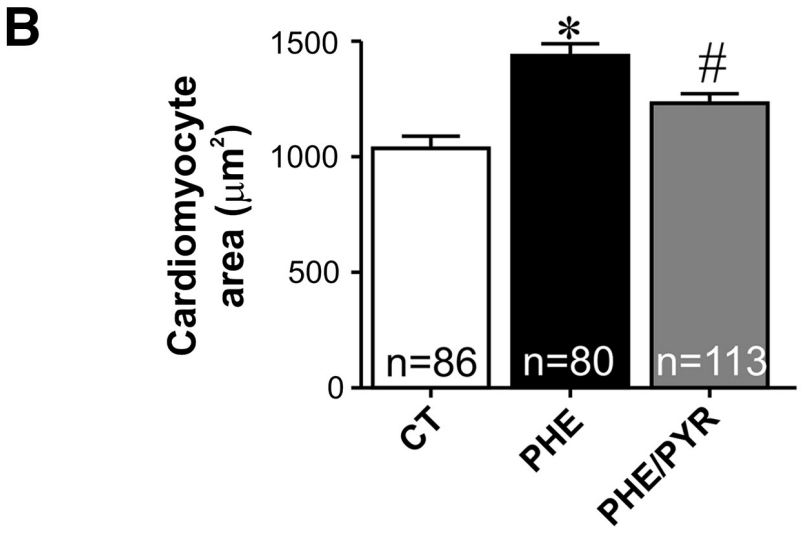
3.5.4 *Cardiomyocyte cholinergic machinery is upregulated by adrenergic stimulation*

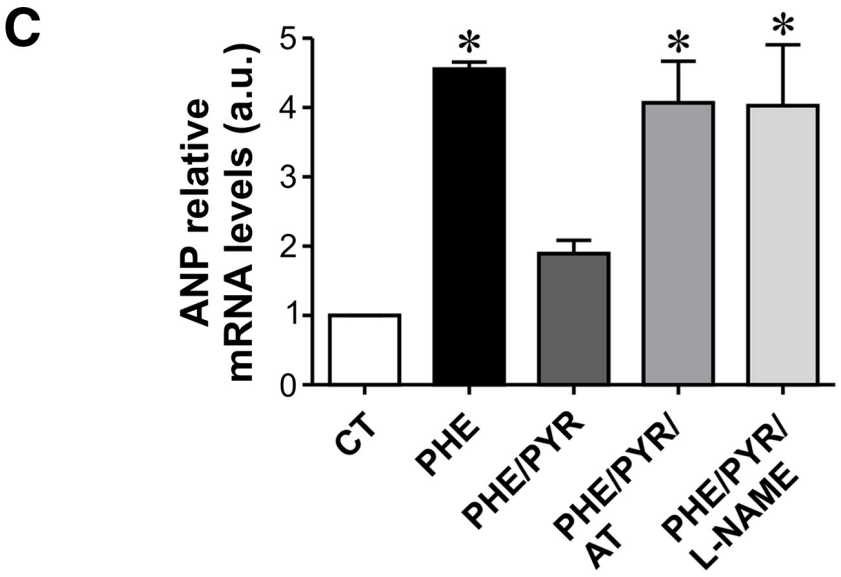
Previous studies using neuronal cells [40] have implicated protein kinase A as an important modulator of VAChT and ChAT mRNA levels, which indicates that adrenergic

Figure 3.12 - Phenylephrine-induced hypertrophy is partially abolished by cholinesterase inhibition in neonatal cardiac myocytes. A. Sample images of α-actinin/DAPI stained neonatal rat cardiomyocytes treated for 48 h with phenylephrine (PHE) or PHE/PYR. Scale bar = 10 μm. **B.** Bar graph showing partial inhibition of PHE induced hypertrophy in PYR-treated neonatal cardiomyocytes. n = number of cells analyzed. **C.** PYR treatment of PHE exposed cardiomyocytes significantly prevents ANP mRNA upregulation. Addition of either atropine or L-NAME blunted PYR effects on ANP mRNA in PHE-exposed cells. n = 3–7 samples from each group. *$p < 0.05$ when compared to control and PHE/PYR group. #$p < 0.05$ when compared to control group.

Figure 3.12 - Phenylephrine-induced hypertrophy is partially abolished by cholinesterase inhibition in neonatal cardiac myocytes.



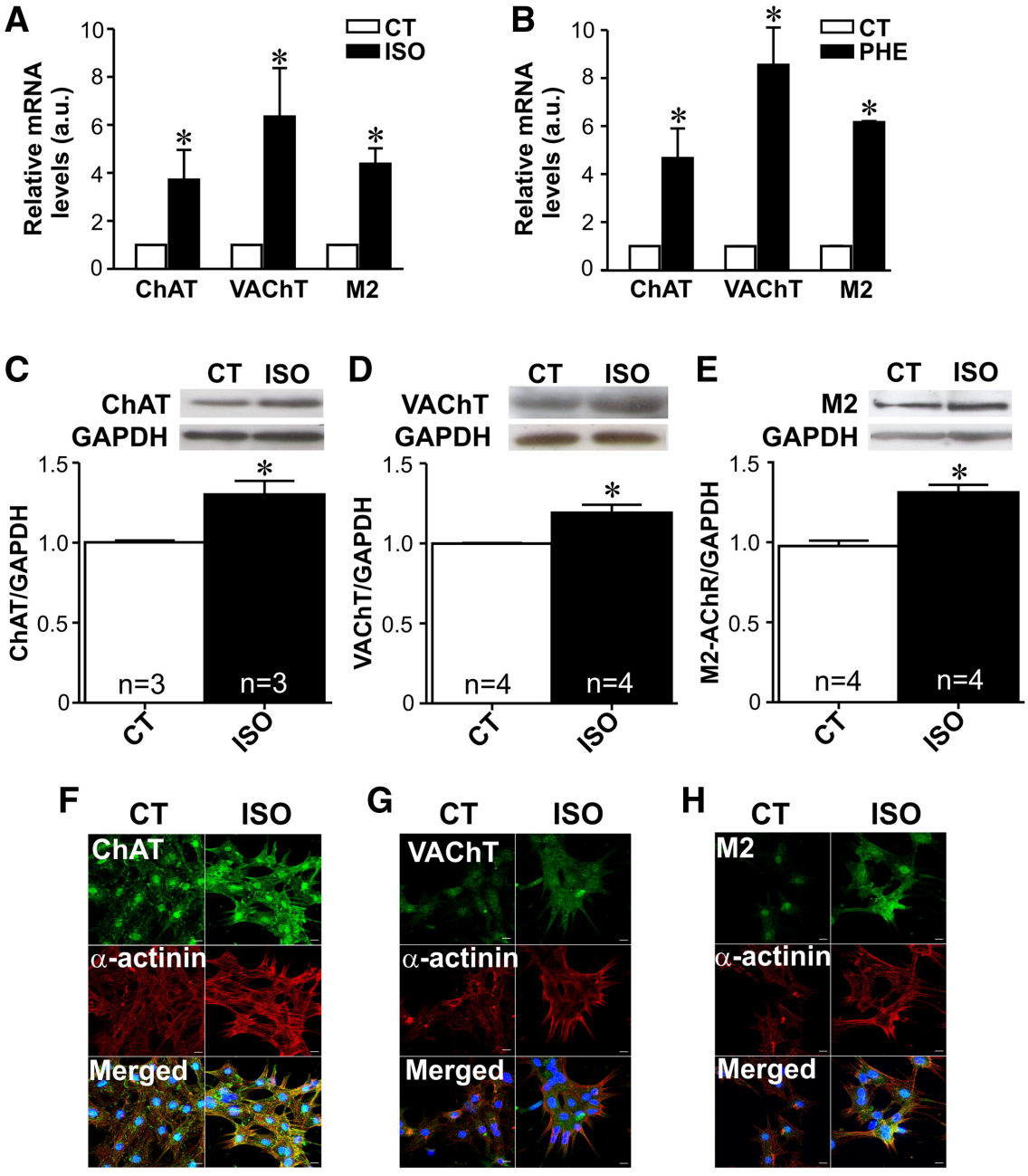




stimulus could regulate expression of cholinergic components. In order to better understand how this intrinsic cholinergic system is modulated and to explore at the mechanistic level how cholinergic signaling counteracts the adrenergic effects, we next assessed the expression levels of cholinergic machinery components in response to isoproterenol and phenylephrine. In neonatal rat cardiomyocytes, ChAT, VAChT and M_2-AChR mRNA were upregulated by isoproterenol (Fig. 3.13A) and phenylephrine (Fig. 3.13B). Similar effect was seen at the protein level for ChAT, VAChT and M_2-AChR on isoproterenol treated cardiomyocytes (Fig. 3.13C-E). We then investigated the effect of isoproterenol on ChAT, VAChT and M_2-AChR localization in neonatal cardiomyocytes by performing immunofluorescence experiments. As shown in Fig. 3.13F, isoproterenol treatment significantly increased ChAT levels at both cytoplasm and nuclei of neonatal cardiomyocytes. Moreover, neonatal cardiomyocytes treated with isoproterenol presented increased VAChT and M_2-AChR cytoplasmic staining (Fig. 3.13G-H). In conclusion, these data show that adrenergic stimulation upregulates the expression levels of cholinergic components in cardiac myocytes.

Figure 3.13 - Adrenergic stimulation upregulates expression of cholinergic machinery in cardiomyocytes. A–B. Either isoproterenol (ISO) or phenyleprine (PHE) upregulates mRNA levels of ChAT, VAChT and M_2-AChR in neonatal cardiac cells. n = 2 to 5 samples from each group. **C–E.** Top, representative Western blot. Bottom, bar graph showing the effect of isoproterenol on ChAT, VAChT and M_2-AChR protein levels. n = number of cardiomyocyte samples analyzed from each group. **F–H.** Immunofluorescence images showing ChAT, VAChT and M_2-AChR changes upon isoproterenol stimulation. The nucleus was stained with DAPI (blue). *$p < 0.05$ when compared to control. Scale bar = 10 μm.

Figure 3.13 - Adrenergic stimulation upregulates expression of cholinergic machinery in cardiomyocytes.



3.6 Discussion

An intrinsic cardiomyocyte cholinergic system has recently been described [17,18]. However, the physiological role of this cardiomyocyte cholinergic machinery and its significance for cardiac function during normal and disease conditions is unknown. Here, we present for the first time evidence that activation of this novel and unexpected machinery has an important role in cardiomyocyte protection, *in vitro*. These data bring into perspective another level of cholinergic control of cardiac function, which goes beyond the cardiac parasympathetic system, and it is intrinsic to ventricular myocytes.

3.6.1 *Localization of cholinergic proteins in cardiomyocytes*

We have extended the observations of two previous reports [17,18], and provide evidence that neonatal and adult cardiomyocytes express prototypical cholinergic markers responsible for ACh synthesis and release. Particularly intriguing is the localization of VAChT to the perinuclear region. Our experiments with vital dye FM1-43 show that this region accumulates recycling vesicles that would be required for the release of ACh. Importantly, cardiomyocytes present all the necessary components required for the exocytosis endocytosis cycle, including SNARE molecules such as SNAP-23, Syntaxin-4, VAMP-1, VAMP-2 and VAMP-3, as well as NSF and its co-factor α-SNAP, the calcium sensor synaptotagmin, and the GTPases Rab8 and Rab4 [25]. Interestingly, SNARE proteins, VAMP-1, VAMP-2, VAMP-3 and syntaxin-4 are found at the perinuclear region of cardiomyocytes [25], supporting our findings. ANP granules are also present in the perinuclear region of cardiomyocytes, suggesting that secretory granules and vesicles are sequestered away from the cellular periphery in these cells. This may represent a way to segregate the exocytosis machinery required to secrete proteins and small molecules from the constant changes in Ca^{2+} levels that occur in cardiomyocytes. It is worth mentioning that Inositol 1,4,5-trisphosphate (InsP3) receptors are found in the perinuclear region [41] where they could provide specific Ca^{2+} signals to activate exocytosis in these cells. However, we cannot discard at the moment the possibility that at least part of the ACh in vesicles is secreted constitutively. The

mechanisms involved in triggering and regulating ACh secretion in cardiomyocytes remain to be identified.

Interestingly, in nerve terminals CHT1 is found mainly in synaptic vesicles and endosomal organelles [42] and only a fraction of the protein is present at the plasma membrane [9]. This does not seem to be the case in cardiomyocytes where the protein is found mainly at the sarcolemma. We note that others have also described similar localization of CHT1 in cardiomyocytes [18].

We have also identified M_2-AChR at the perinuclear region of cardiac cells in addition to its localization at the T-tubular system. This finding is in agreement with previous data showing the presence of the G_i subunit at the nuclear periphery of ventricular myocytes [43]. In addition, β-adrenergic receptors have also been found at these nuclear structures [43]. Whether this intracellular pool of receptors represents newly-synthesized and recycling receptors or represents functional receptors is still unknown. The possibility that ACh may play unanticipated roles in regulating gene transcription in the cardiac cells by activating nuclear M_2 receptors is intriguing. The functional consequences of this nuclear receptor activation, however, have yet to be elucidated.

3.6.2 *ACh release by cardiomyocytes depends on VAChT activity*

Although others have shown that ACh is released by cardiomyocytes [17,18], our data extend these findings by demonstrating the functionality of this phenomenon. By using the production of NO as a biosensor for released ACh, and by using the cardiomyocytes themselves as a detection sensor, we showed that inhibition of cholinesterase significantly increases DAF fluorescence i.e. increases NO levels. This effect is similar to that observed when exogenous ACh is added to freshly isolated ventricular myocytes and it is completely abolished by a muscarinic antagonist and by a specific inhibitor of the CHT1. CHT1 is required in nerve terminals for the synthesis of ACh [44], and our data suggest it has similar roles in cardiomyocytes. It is particularly interesting that HC-3 can

block the effects of pyridostigmine as it suggests that no other sources of choline can be utilized in order to generate ACh in cardiac cells.

Our data indicate that ACh secretion by cardiomyocytes requires vesicular mechanisms since ventricular myocytes obtained from mice that express substantially less VAChT (VAChT knockdown mice) showed decreased availability of ACh for activation of DAF fluorescence. This result is consistent with our finding that some of the effects of pyridostigmine or neostigmine are inhibited by vesamicol, a selective VAChT inhibitor. VAChT is part of an ancient transport machinery [45] and, as long as it is present in acidic vesicles, it will be able to transport ACh [46]. Hence, taken together, these experiments provide strong evidence that ACh release by cardiomyocytes depends on VAChT activity. Moreover, our data show that observed effects of pyridostigmine occur via preservation of ACh. It is unlikely that pyridostigmine would have off target effects as similar results were also observed with a second cholinesterase inhibitor, neostigmine, and in cells transfected with a siRNA targeted to AChE. Moreover anti-hypertrophic effects of pyridostigmine were not observed in the absence of ACh transport to vesicles.

3.6.3 *ACh secreted by cardiomyocytes prevents adrenergic hypertrophic effects*

Since proteins involved in the synthesis and release of ACh are found in cardiomyocytes, this raises the question as to whether this intrinsic cholinergic system has any direct functional role in regulating cardiomyocyte activity. Considering that we used a cholinesterase inhibitor to observe the effect of cardiomyocyte-derived ACh, it could be argued that this local cholinergic machinery is not essential to cardiac function. However, it should be noted that although parasympathetic innervations in the ventricle are scarce [47], muscarinic receptors are expressed in ventricular myocytes [48]. Therefore, we speculate that this intrinsic cardiomyocyte cholinergic signaling is part of an adaptive defense mechanism against cardiomyocyte insults. This is further supported by our finding that ventricular myocytes from VAChT knockdown mice present a Ca^{2+} signaling dysfunction that is restored upon pyridostigmine administration, *in vivo* [10], indicating that lack of ACh release has profound effects on ventricular myocytes.

While our data indicates that basal ACh release by cardiomyocytes is low, it is plausible that under certain circumstances ACh release can increase, and exerts protective effects. Modulation of ChAT, VAChT and CHT1 expression levels has already been demonstrated in neuronal cells [49,50]. Accordingly, cAMP leads to a significant increase in ChAT and VAChT mRNA levels in SN56 cells. Here, we show for the first time that chronic adrenergic stimulation upregulates expression levels of cholinergic components VAChT, ChAT and M_2-AChR in rat cardiac cells, suggesting that ACh release is increased under these conditions. Supporting this assumption, we show that isoproterenol-induced increase in cell surface area is exacerbated when VAChT activity, and consequently ACh release, is inhibited. Together, these findings unravel a role for intrinsic ACh as an *in situ* negative feedback signal that counteracts adrenergic effects.

At the mechanistic level, the ability of pyridostigmine to prevent the increase in Ca^{2+} transient induced by prolonged treatment with isoproterenol is of major importance since alterations in Ca^{2+} may act as a trigger for cardiac remodeling. Accordingly, ACh secreted locally by cardiomyocytes can act in a paracrine/autocrine manner to antagonize the effects of hyperadrenergic stimulation. Taking this into consideration, we speculate that under certain circumstances local cardiac cholinergic system could contribute to the protective effects of the parasympathetic nervous system in the heart. This possibility must be considered in future studies of cardiovascular pathology in which treatment with cholinesterase inhibitor is being evaluated, *in vivo*.

The role of non-neuronal ACh synthesis and release machinery in other cell types has also begun to be elucidated. For example, in the immune system, ACh synthesized by a population of T lymphocytes has been recently shown to be required for the function of the cholinergic anti-inflammatory system [51]. In humans, non-neuronal ACh in α cells in the pancreas is important for the secretion of insulin [52]. Here we also show a putative role for cholinergic signaling present in cardiomyocytes by opposing the hypertrophic effects of adrenergic stimulation. It is worth mentioning that the only source of ACh in our experiments is the cardiac cell, as evidenced by the absence of neuronal cells in our culture conditions. Moreover, the use of a broad range of pharmacological

agents that interfere with the synthesis and storage of ACh in vesicles support the notion that cardiomyocytes have adapted mechanisms used by neurons to secrete ACh. Given that ACh is an ancient and widespread chemical used for cell-cell communication, it should not come as a surprise to find so many distinct non-neuronal roles for ACh. Future experiments, using conditional knockout mice [23,53] will be necessary to further define the role of this non-neuronal cholinergic system in cardiomyocytes in physiological and pathological alterations in the heart *in vivo*.

3.6.4 *Conclusion*

Taken together, our results show that cardiomyocytes express neuronal proteins required for ACh synthesis and release and are capable of secreting ACh. In these cells, VAChT is found mainly at the perinuclear region, where it extensively colocalizes with recycling vesicles that are required for ACh release. ACh secretion by cardiomyocytes depends on both VAChT activity and choline reuptake by CHT1, as observed in neurons. In addition, we show that cardiomyocyte secreted ACh impairs adrenergic hypertrophic signaling, indicating that ACh acting autocrinally has a protective role.

3.7 Acknowledgments

This work was supported by grants from NIH R03TW008425 from the Fogarty International Center (SG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (SG, CG, RRR), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (SG, CG and RRR), PRONEX APQ-00746-09 (SG), the Heart and Stroke Foundation of Ontario (MAMP and VFP) and CIHR (RG, VFP and MAMP). C. Rocha-Resende is a recipient of the CNPq PhD fellowship at the Post-graduation Program in Biological Science: Physiology and Pharmacology at UFMG. Ashbeel Roy is a recipient of the Queen Elizabeth II Scholarship in Science and Technology (QEIISST). Robert Gros is supported by a New Investigator Award from the Heart and Stroke Foundation of Canada.

3.8 References

[1] Dhein S, van Koppen CJ, Brodde OE. Muscarinic receptors in the mammalian heart. Pharmacol Res 2001;44:161–82.

[2] Loffelholz K, Pappano AJ. The parasympathetic neuroeffector junction of the heart. Pharmacol Rev 1985;37:1–24.

[3] Kent KM, Epstein SE, Cooper T, Jacobowitz DM. Cholinergic innervation of the canine and human ventricular conducting system. Anatomic and electrophysiologic correlations. Circulation 1974;50:948–55.

[4] Nagata K, Ye C, Jain M, Milstone DS, Liao R, Mortensen RM. Galpha(i2) but not Galpha(i3) is required for muscarinic inhibition of contractility and calcium currents in adult cardiomyocytes. Circ Res 2000;87:903–9.

[5] Katare RG, Ando M, Kakinuma Y, Arikawa M, Yamasaki F, Sato T. Differential regulation of TNF receptors by vagal nerve stimulation protects heart against acute ischemic injury. J Mol Cell Cardiol 2010;49:234–44.

[6] de Castro BM, Pereira GS, Magalhaes V, Rossato JI, De JX, Martins-Silva C. Reduced expression of the vesicular acetylcholine transporter causes learning deficits in mice. Genes Brain Behav 2009;8:23–35.

[7] de Castro BM, De JX, Martins-Silva C, Lima RD, Amaral E, Menezes C, et al. The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol 2009;29:5238–50.

[8] Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, et al. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron 2006;51:601–12.

[9] Ribeiro FM, Black SA, Prado VF, Rylett RJ, Ferguson SS, Prado MA. The “ins” and “outs” of the high-affinity choline transporter CHT1. J Neurochem 2006;97:1–12.

[10] Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, et al. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol 2010;30:1746–56.

[11] English BA, Appalsamy M, Diedrich A, Ruggiero AM, Lund D, Wright J, et al. Tachycardia, reduced vagal capacity, and age-dependent ventricular dysfunction arising from diminished expression of the presynaptic choline transporter. Am J Physiol Heart Circ Physiol 2010;299:H799–810.

[12] LaCroix C, Freeling J, Giles A, Wess J, Li YF. Deficiency of M2 muscarinic acetylcholine receptors increases susceptibility of ventricular function to chronic adrenergic stress. Am J Physiol Heart Circ Physiol 2008;294:H810–20.

[13] Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation 2004;109:120–4.

[14] Freeling J, Wattier K, LaCroix C, Li YF. Neostigmine and pilocarpine attenuated tumour necrosis factor alpha expression and cardiac hypertrophy in the heart with pressure overload. Exp Physiol 2008;93:75–82.

[15] Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. J Clin Invest 2010;120:408–21.

[16] Huston JM, Tracey KJ. The pulse of inflammation: heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy. J Intern Med 2011;269:45–53.

[17] Kakinuma Y, Akiyama T, Sato T. Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J 2009;276:5111–25.

[18] Rana OR, Schauerte P, Kluttig R, Schroder JW, Koenen RR, Weber C, et al. Acetylcholine as an age-dependent non-neuronal source in the heart. Auton Neurosci

2010;156:82–9.

[19] Guatimosim S, Amaya MJ, Guerra MT, Aguiar CJ, Goes AM, Gomez-Viquez NL, et al. Nuclear Ca2+ regulates cardiomyocyte function. Cell Calcium 2008;44:230–42.

[20] Mohler PJ, Schott JJ, Gramolini AO, Dilly KW, Guatimosim S, duBell WH, et al. Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. Nature 2003;421:634–9.

[21] Aguiar CJ, Andrade VL, Gomes ER, Alves MN, Ladeira MS, Pinheiro AC, et al. Succinate modulates Ca(2+) transient and cardiomyocyte viability through PKAdependent pathway. Cell Calcium 2010;47:37–46.

[22] Guatimosim S, Sobie EA, dos Santos CJ, Martin LA, Lederer WJ. Molecular identification of a TTX-sensitive Ca(2+) current. Am J Physiol Cell Physiol 2001;280: C1327–39.

[23] Guzman MS, De JX, Raulic S, Souza IA, Li AX, Schmid S, et al. Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behavior by cholinergic-glutamatergic co-transmission. PLoS Biol 2011;9:e1001194.

[24] Guatimosim C, Romano-Silva MA, Gomez MV, Prado MA. Use of fluorescent probes to follow membrane traffic in nerve terminals. Braz J Med Biol Res 1998;31: 1491–500.

[25] FerlitoM, FultonWB, Zauher MA,Marban E, Steenbergen C, Lowenstein CJ. VAMP-1, VAMP-2, and syntaxin-4 regulate ANP release from cardiac myocytes. J Mol Cell Cardiol 2010;49:791–800.

[26] Gill SK, Bhattacharya M, Ferguson SS, Rylett RJ. Identification of a novel nuclear localization signal common to 69- and 82-kDa human choline acetyltransferase. J Biol Chem 2003;278:20217–24.

[27] Kitakaze M, Node K, Komamura K, Minamino T, Inoue M, Hori M, et al. Evidence for nitric oxide generation in the cardiomyocytes: its augmentation by hypoxia. J Mol

Cell Cardiol 1995;27:2149–54.

[28] Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. J Mol Cell Cardiol 2011;51:288–98.

[29] Volz A, Piper HM, Siegmund B, Schwartz P. Longevity of adult ventricular rat heart muscle cells in serum-free primary culture. J Mol Cell Cardiol 1991;23:161–73.

[30] Zaruba MM, Field LJ. The mouse as a model system to study cardiac regeneration. Drug Discov Today Dis Models 2008;5:165–71.

[31] Liu Y, Edwards RH. Differential localization of vesicular acetylcholine and monoamine transporters in PC12 cells but not CHO cells. J Cell Biol 1997;139:907–16.

[32] Varoqui H, Meunier FM, Meunier FA, Molgo J, Berrard S, Cervini R, et al. Expression of the vesicular acetylcholine transporter in mammalian cells. Prog Brain Res 1996;109:83–95.

[33] Nyquist-Battie C, Hagler KE, Love S. Reduced levels of globular and asymmetric forms of acetylcholinesterase in rat left ventricle with pressure overload hypertrophy. Life Sci 1994;55:653–9.

[34] Peters CG, Miller DF, Giovannucci DR. Identification, localization and interaction of SNARE proteins in atrial cardiac myocytes. J Mol Cell Cardiol 2006;40: 361–74.

[35] Morisco C, Zebrowski DC, Vatner DE, Vatner SF, Sadoshima J. Beta-adrenergic cardiac hypertrophy is mediated primarily by the beta(1)-subtype in the rat heart. J Mol Cell Cardiol 2001;33:561–73.

[36] Sucharov CC, Mariner PD, Nunley KR, Long C, Leinwand L, Bristow MR. A beta1-adrenergic receptor CaM kinase II-dependent pathway mediates cardiac myocyte fetal gene induction. Am J Physiol Heart Circ Physiol 2006;291:H1299–308.

[37] Zou Y, Yao A, Zhu W, Kudoh S, Hiroi Y, Shimoyama M, et al. Isoproterenol activates extracellular signal-regulated protein kinases in cardiomyocytes through

calcineurin. Circulation 2001;104:102–8.

[38] Lanzafame AA, Christopoulos A, Mitchelson F. Cellular signaling mechanisms for muscarinic acetylcholine receptors. Receptors Channels 2003;9:241–60.

[39] Fleming JW, Wisler PL, Watanabe AM. Signal transduction by G proteins in cardiac tissues. Circulation 1992;85:420–33.

[40] Castell X, Cheviron N, Barnier JV, Diebler MF. Exploring the regulation of the expression of ChAT and VAChT genes in NG108-15 cells: implication of PKA and PI3K signaling pathways. Neurochem Res 2003;28:557–64.

[41] Luo D, Yang D, Lan X, Li K, Li X, Chen J, et al. Nuclear Ca2+ sparks and waves mediated by inositol 1,4,5-trisphosphate receptors in neonatal rat cardiomyocytes. Cell Calcium 2008;43:165–74.

[42] Ribeiro FM, ves-Silva J, Volknandt W, Martins-Silva C, Mahmud H, Wilhelm A, et al. The hemicholinium-3 sensitive high affinity choline transporter is internalized by clathrin-mediated endocytosis and is present in endosomes and synaptic vesicles. J Neurochem 2003;87:136–46.

[43] Boivin B, Lavoie C, Vaniotis G, Baragli A, Villeneuve LR, Ethier N, et al. Functional beta-adrenergic receptor signalling on nuclear membranes in adult rat and mouse ventricular cardiomyocytes. Cardiovasc Res 2006;71:69–78.

[44] Ferguson SM, Bazalakova M, Savchenko V, Tapia JC, Wright J, Blakely RD. Lethal impairment of cholinergic neurotransmission in hemicholinium-3-sensitive choline transporter knockout mice. Proc Natl Acad Sci U S A 2004;101:8762–7.

[45] Bravo DT, Kolmakova NG, Parsons SM. New transport assay demonstrates vesicular acetylcholine transporter has many alternative substrates. Neurochem Int 2005;47:243–7.

[46] Prado MA, Reis RA, Prado VF, de Mello MC, Gomez MV, de Mello FG. Regulation of acetylcholine synthesis and storage. Neurochem Int 2002;41:291–9.

[47] Hancock JC, Hoover DB, Hougland MW. Distribution of muscarinic receptors and acetylcholinesterase in the rat heart. J Auton Nerv Syst 1987;19:59–66.

[48] Wei JW, Sulakhe PV. Regional and subcellular distribution of myocardial muscarinic cholinergic receptors. Eur J Pharmacol 1978;52:235–8.

[49] Berse B, Blusztajn JK. Coordinated up-regulation of choline acetyltransferase and vesicular acetylcholine transporter gene expression by the retinoic acid receptor alpha, cAMP, and leukemia inhibitory factor/ciliary neurotrophic factor signaling pathways in a murine septal cell line. J Biol Chem 1995;270:22101–4.

[50] Brock M, Nickel AC, Madziar B, Blusztajn JK, Berse B. Differential regulation of the high affinity choline transporter and the cholinergic locus by cAMP signaling pathways. Brain Res 2007;1145:1–10.

[51] Rosas-BallinaM, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science 2011;334:98–101.

[52] Rodriguez-Diaz R, Dando R, Jacques-Silva MC, Fachado A, Molina J, Abdulreda MH, et al. Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans. Nat Med 2011;17:888–92.

[53] Martins-Silva C, De JX, Guzman MS, Lima RD, Santos MS, Kushmerick C, et al. Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PLoS One 2011;6:e17611.

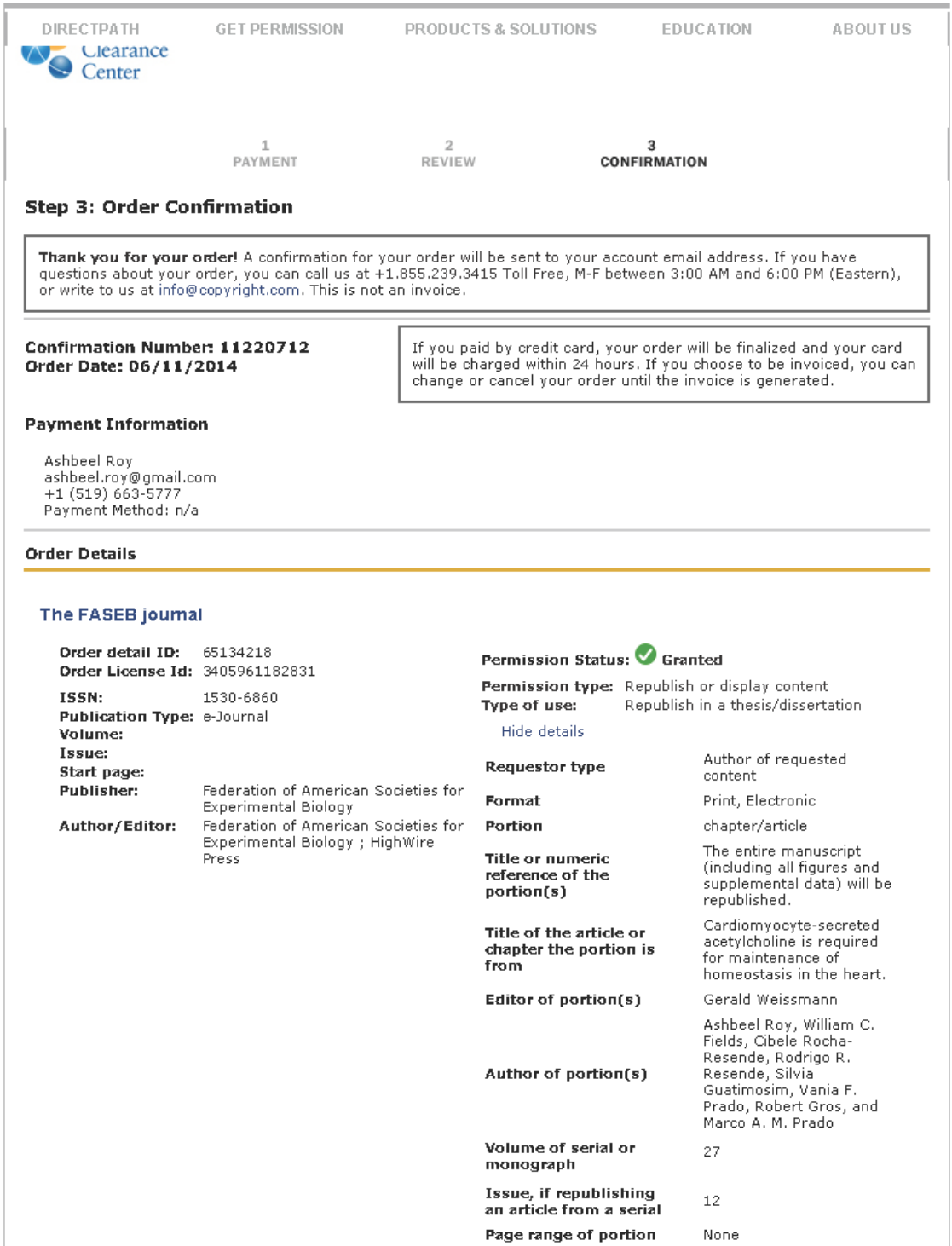
Chapter 4

CARDIOMYOCYTE-SECRETED ACETYLCHOLINE IS REQUIRED FOR MAINTENANCE OF HOMEOSTASIS IN THE HEART

A version of this chapter has previously been published: Roy A, Fields WC, Rocha-Resende C, Resende RR, Guatimosim S, Prado VF, Gros R, Prado MA. (2013). Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. ***FASEB J.*** 2013 Dec;27(12):5072-82.

4 Chapter 4

4.1 Copyright Permission



DIRECTPATH GET PERMISSION PRODUCTS & SOLUTIONS EDUCATION ABOUT US

Clearance Center

1 PAYMENT 2 REVIEW 3 CONFIRMATION

Step 3: Order Confirmation

Thank you for your order! A confirmation for your order will be sent to your account email address. If you have questions about your order, you can call us at +1.855.239.3415 Toll Free, M-F between 3:00 AM and 6:00 PM (Eastern), or write to us at info@copyright.com. This is not an invoice.

Confirmation Number: 11220712
Order Date: 06/11/2014

If you paid by credit card, your order will be finalized and your card will be charged within 24 hours. If you choose to be invoiced, you can change or cancel your order until the invoice is generated.

Payment Information

Ashbeel Roy
ashbeel.roy@gmail.com
+1 (519) 663-5777
Payment Method: n/a

Order Details

The FASEB journal

Order detail ID:	65134218
Order License Id:	3405961182831
ISSN:	1530-6860
Publication Type:	e-Journal
Volume:	
Issue:	
Start page:	
Publisher:	Federation of American Societies for Experimental Biology
Author/Editor:	Federation of American Societies for Experimental Biology ; HighWire Press

Permission Status: Granted
Permission type: Republish or display content
Type of use: Republish in a thesis/dissertation

Hide details

Requestor type	Author of requested content
Format	Print, Electronic
Portion	chapter/article
Title or numeric reference of the portion(s)	The entire manuscript (including all figures and supplemental data) will be republished.
Title of the article or chapter the portion is from	Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart.
Editor of portion(s)	Gerald Weissmann
Author of portion(s)	Ashbeel Roy, William C. Fields, Cibele Rocha-Resende, Rodrigo R. Resende, Silvia Guatimosim, Vania F. Prado, Robert Gros, and Marco A. M. Prado
Volume of serial or monograph	27
Issue, if republishing an article from a serial	12
Page range of portion	None

Publication date of portion	Dec. 2013
Rights for	Main product
Duration of use	Life of current edition
Creation of copies for the disabled	no
With minor editing privileges	no
For distribution to	Worldwide
In the following language(s)	Original language of publication
With incidental promotional use	no
Lifetime unit quantity of new product	0 to 499
Made available in the following markets	Education
The requesting person/organization	Ashbeel Roy
Order reference number	None
Author/Editor	Ashbeel Roy
The standard identifier	Thesis/dissertation
Title	The implications of altered cholinergic signaling in cardiac health and disease
Publisher	University of Western Ontario
Expected publication date	Aug 2014
Estimated size (pages)	300

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. More info **$ 0.00**

This is not an invoice.

Total order items: 1

Order Total: 0.00 USD

4.2 Chapter Summary

Heart activity and long-term function are regulated by the sympathetic and parasympathetic branches of the nervous system. Parasympathetic neurons have received increased attention recently because acetylcholine (ACh) has been shown to play protective roles in heart disease. However, parasympathetic innervation is sparse in the heart, raising the question of how cholinergic signalling regulates cardiomyocytes. We hypothesized that non-neuronal secretion of ACh from cardiomyocytes plays a role in cholinergic regulation of cardiac activity. To test this possibility, we eliminated secretion of ACh exclusively from cardiomyocytes by targeting the vesicular acetylcholine transporter (VAChT). We find that lack of cardiomyocyte-secreted ACh disturbs the regulation of cardiac activity and causes cardiomyocyte remodelling. Mutant mice present normal hemodynamic parameters under non-stressful conditions; however, following exercise, their heart rate response is increased. Moreover, hearts from mutant mice present increased oxidative stress, altered calcium signalling, remodelling and hypertrophy. Hence, without cardiomyocyte-derived ACh secretion, hearts from mutant mice show signs of imbalanced autonomic activity consistent with decreased cholinergic drive. These unexpected results suggest that cardiomyocyte-derived ACh is required for maintenance of cardiac homeostasis and regulates critical signalling pathways necessary to maintain normal heart activity. We propose that this non-neuronal source of ACh boosts parasympathetic cholinergic signalling to counterbalance sympathetic activity regulating multiple aspects of heart physiology.

4.3 Introduction

Acetylcholine released by parasympathetic nerves regulates the minute to minute changes in heart rate and contractility required for proper cardiovascular function via muscarinic receptors, opposing the activity of the sympathetic nervous system [1]. In addition to regulating atrial activity, ACh plays multiple roles in ventricular function [2, 3], notwithstanding limited parasympathetic innervation in ventricular regions [4, 5]. The exact mechanisms by which ACh can have such widespread effects, despite somewhat limited parasympathetic innervation in regions other than the atria, are not fully understood. Interestingly, in the pancreas [6] as well as the immune system [7, 8], non-neuronal sources of ACh secretion have recently been shown to play a role in regulating insulin secretion [6] and the cholinergic anti-inflammatory system [9, 10], respectively. However, there is limited information regarding the contribution of non-neuronal ACh for autonomic regulation of other bodily functions, *in vivo*.

Recent experiments have indicated that cultured cardiomyocytes, similar to pancreatic alpha cells [6] and lymphocytes [7], can express the enzymatic machinery required for ACh synthesis [11, 12]. Moreover, secretion of ACh from cultured cardiomyocytes depends on the activity of the vesicular acetylcholine transporter (VAChT) [12]. However, whether this non-neuronal source of ACh plays a physiological role in the regulation of heart activity or function is unknown. Here, we used the Cre-loxP system to eliminate VAChT exclusively in cardiomyocytes and test for potential roles for cardiomyocyte-secreted ACh in heart activity. We found that this novel form of cellular communication is required for physiological regulation of heart size and stress levels, likely by maintaining high levels of ACh at synaptic junctions. We also discovered that the recovery of heart rate following exercise or stress is disturbed in the absence of this non-neuronal source of ACh, suggesting widespread effects of non-neuronal cardiomyocyte-derived ACh. Our study provides a novel mechanism for autocrine/paracrine regulation of cardiomyocytes by non-neuronal ACh secretion.

4.4 Materials and Methods

4.4.1 Animals

Transgenic mice expressing Cre under the control of the cardiac-specific murine alpha myosin-heavy chain promoter (Myh6-cre) were obtained from JAX Laboratories (B6.FVB-Tg(Myh6-cre)2182Mds/J, stock #011038) and bred to VAChT-floxed (flanked by lox P) mice ($VAChT^{flox/flox}$) [13]. $VAChT^{flox/flox}$ mice have been backcrossed 5 times to C57BL6/j mice as described [13]. $VAChT^{flox/flox}$ mice are not different from wild-type mice [14]. Littermates ($VAChT^{WT/flox,Myh6\text{-}cre+}$ X $VAChT^{WT/flox,Myh6\text{-}cre-}$) were crossed to generate $VAChT^{flox/flox,Myh6\text{-}cre+}$ and $VAChT^{flox/flox,Myh6\text{-}cre-}$. F2 littermates were then bred to generate mice used for this study. $VAChT^{flox/flox,Myh6\text{-}cre-}$ littermates were used as controls.

In order to generate the cardiomyocyte-specific ChAT knockout mice, Myh6-cre mice were bred to ChAT-floxed mice [15] obtained from JAX Laboratories (B6.129-*Chattm1Jrs*/J; Jackson Laboratory; $ChAT^{flox/flox}$) [13]. Littermates ($ChAT^{WT/flox,Myh6\text{-}cre+}$ X $ChAT^{WT/flox,Myh6\text{-}cre-}$) were crossed to generate $ChAT^{flox/flox,Myh6\text{-}cre+}$ and $ChAT^{flox/flox,Myh6\text{-}cre-}$.

The Rosa-EYFP strain (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J; JAX Laboratories; stock # 006148) was used as a Cre reporter. Animals were maintained and cared for according to approved animal protocol at the University of Western Ontario (2008-127) and following Canadian Council on Animal Care. Only male mice were used for adult cardiomyocyte isolation and all *in vivo* experiments. Neonatal cardiomyocytes were isolated and cultured from mice of both genders.

4.4.2 Neonatal cardiomyocyte culture

Neonatal cardiomyocytes were isolated as previously described [16]. Briefly, cardiac cells were plated in dishes containing M199 medium supplemented with 100 units/mL

penicillin, 100 μg/mL streptomycin, 10% FBS and 2 mmol/L L-glutamine. Cytosine-D-arabinofuranoside (ARA-c) (20 μg/mL) was used to prevent growth of fibroblasts. For hypertrophy studies, cardiac cells at day 4 in culture were incubated with vesamicol (VES, 5 μM) or hemicholium-3 (HC-3, 10 μM) for 48 h and then used for immunofluorescence or qPCR analyses.

4.4.3 qPCR/RT-PCR

RNA was extracted from isolated cardiomyocytes and cDNA was synthesized as previously described [17]. A brain sample was used as a positive control for VAChT and a non-template reaction was used as a negative control. qPCR for ANP and GRK5 was performed as previously described [17].

4.4.4 Immunoblotting

Isolated adult cardiomyocytes were lysed using ice-cold modified RIPA buffer. 80 μg of protein was separated using SDS-PAGE and PVDF membranes were probed with anti-VAChT antibody (1:200; Synaptic Systems). α-actinin (1:2000; Sigma-Aldrich) was used as a loading control.

4.4.5 Immunostaining

Adult cardiomyocytes were subjected to immunofluorescence protocol as previously described [12]. Cells were incubated with one of the following antibodies: anti-VAChT (1:50; Synaptic Systems), anti-ChAT (1:100; Abcam), anti-CHT1 (1:500; kindly supplied by Dr. R. Jane Rylett) [18], anti-ANP (1:200; Abcam) or anti-GRK5 (1:100; Santa Cruz Biotechnologies). The cells were co-labelled with α-actinin (1:200; Sigma-Aldrich). α-actinin labeled cells were used to measure cardiomyocyte cell surface area. Images were acquired using either the Leica SP5 II or Zeiss LSM 510 Meta confocal system (63x objective, 488 nm Argon laser and 546 nm HeNe laser were used for excitation of fluorophores).

4.4.6 Measurement of ACh secretion (Fluorometric Assay)

Transmitter release was measured using the Choline/ACh Quantification Kit (Biovision) as previously described [19]. Briefly, cultured media was collected from $VAChT^{flox/flox}$ and $VAChT^{Myh6\text{-}Cre\text{-}flox/flox}$ cardiomyocytes and incubated with either 100 µM pyridostigmine bromide (Sigma-Aldrich; P9797) or 100 µM pyridostigmine bromide and 1 µM vesamicol hydrochloride (Sigma-Aldrich; V100) at 37ºC for 4 hours. Cultured media was collected and centrifuged at 10,000 RPM for 5 minutes at 4ºC. The resulting supernatant was collected and filtered using a 0.2 µm Acrodisc Syringe Filter (PALL Life Sciences) and placed on ice. ACh concentration was determined using the fluorometric Choline/ACh Quantification Kit (Ex=535, Em=590 nm). Each sample was assayed in duplicate and experiments were conducted at least 4 times using separate cultures.

4.4.7 Measurement of ACh secretion (HPLC-electrochemical detection)

Cultured neonatal cardiomyocytes from $VAChT^{flox/flox}$ and $VAChT^{Myh6\text{-}Cre\text{-}flox/flox}$ were treated with 100 µM pyridostigmine bromide (Sigma Aldrich; P9797) and incubated for 4 hours at 37ºC. It has previously been reported that both subacute treatment with pyridostigmine [20] as well as acute exposure to stress [21] can increase the transcription of acetylcholinesterase (AChE) in the brain. However, *in vitro* studies have confirmed that pyridostigmine is a very potent AChE inhibitor (IC_{50} = 0.33 µM for inhibition of erythrocyte AChE activity) [22]. As such, 100 µM pyridostigmine was used in our experiments to ensure the inhibition of virtually all AChE activity throughout the experiment. Cultured medium was collected and centrifuged at 13,200 RPM for 5 minutes at 4ºC. The supernatant was filtered through a 0.2 µM Acrodisc Syringe Filter (PALL Life Sciences; PN=4602) and injected into an ESA UltiMATE 3000 system with a Choulochem electrochemical detector. The sequence was run using the following components and parameters: Flow rate: 0.300 mL/min; Injection volume: 20 µL; Cell potential: 275 mV; Column: MGII CAPCELL PAK C18 column (Shiseido; Cat. No.=

92461); Column temperature: 40ºC; ACh post-column solid phase reactor (Thermo Scientific; 70-0640A).

4.4.8 NO measurement

Neonatal cardiomyocytes from control and cVAChT mice were used to measure NO production as previously described [12]. Cells were incubated with either carbachol (10 μM) or pyridostigmine (100 μM). Images were acquired using the Leica SP5 II confocal system and analyzed using ImageJ software.

4.4.9 Immunohistochemistry

Hearts were excised and fixed using 4% PFA. IHC was performed on slices as previously described [23]. Slices were incubated with either anti-VAChT (1:200; Synaptic Systems) or anti-CHT1 (1:200) [18] to visualize intracardiac ganglia and terminals respectively.

4.4.10 Heart rate and BP recordings

Both heart rate and blood pressure were recorded from conscious animals using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA) as previously described [24].

4.4.11 Electrocardiography

ECGs were recorded using radio telemeters as previously described [3]. HR was recorded continuously over 24h to obtain baseline recordings. Additionally, heart rates were recorded in the home cages immediately following IP injection of saline or following an acute exercise routine (ramp up from 5 m/min to 15 m/min over 60 s, followed by 180 s at 15 m/min).

4.4.12 Cardiomyocyte morphometry

In situ cardiomyocyte cell surface area was measured as previously described [3].

4.4.13 ROS measurement

ROS levels were measured using the MitoSOX Red superoxide indicator (Invitrogen) as previously described [17].

4.4.14 Protein oxidation measurement

Protein was isolated from whole hearts using ice-cold modified RIPA buffer, separated using SDS-PAGE and transferred onto PVDF membrane. The levels of oxidatively modified proteins were analyzed in control and cVAChT hearts using the OxyBlot Protein Oxidation Detection Kit (Millipore) following the manufacturer's directions.

4.4.15 Indirect calorimetry, activity and inactivity

These experiments were performed using the CLAMS metabolic chamber system (Columbus Instruments) as previously described [25, 26]. VO_2, VCO_2, food and water intake, activity and inactivity were measured. Respiratory exchange ratio and energy expenditure/heat were calculated within the Oxymax software. Periods of inactivity (sleep) were obtained using the sleep detection algorithms available within the Oxymax software.

4.4.16 Preparation of siRNA

Potential target sites within the VAChT gene were selected and then searched with NCBI Blast to confirm specificity for the transporter. The siRNA for VAChT was prepared as previously described by our group [27]. The sense and antisense oligonucleotides of siRNA were respectively, as follows: 5'-GGAGCAGGGAGGCAGAAGAAGCTGT-3' and 5'-ACAGCTTCTTCTGCCTCCCTGCTCCAT-3'. For siRNA studies, neonatal

cardiomyocyte cultures were transfected at day 4 with 100 nM of siRNA 48 h prior to measurements. The cells were then used for immunofluorescence, immunobloting or qPCR analyses.

4.4.17 Cardiomyocyte isolation and Ca^{2+} recordings

Adult cardiomyocytes were isolated and calcium transients were recorded as previously described [3, 17] using line-scan imaging on a Zeiss LSM 510 Meta confocal microscope. Images were processed and Ca^{2+} recordings were analyzed using ImageJ software.

4.4.18 Hemodynamic measurements

Invasive left ventricular (LV) hemodynamic measurements were obtained under baseline as well as following administration of isoproterenol (0.5 µg intraperitoneally [i.p.]) using a Millar Mikro-tip pressure transducer (Millar Instruments, Houston, TX, USA) as previously described [3]. All the LV parameters were obtained using the PowerLab Chart Analysis software (AD Instruments, Colorado Springs, CO, USA).

4.4.19 Statistical analyses

Results for experiments are provided as mean ± SEM. Student's t-test, one-way ANOVA with a Tukey's post-hoc test or two-way ANOVA were used to assess statistical differences between experimental groups using as required using Graphpad or SigmaStat. $p<0.05$ was considered statistically significant.

4.5 Results

4.5.1 Generation of cardiomyocyte-specific VAChT null mice

Previous experiments indicated that, in cultured neonatal cardiomyocytes, ACh secretion is dependent on the activity of VAChT (12), a transporter that is critical for ACh storage in nerve-endings [28, 29]. In order to selectively interfere with secretion of ACh from cardiomyocytes *in vivo* we generated a cardiomyocyte-specific VAChT knockout mouse line ($VAChT^{Myh6\text{-}Cre\text{-}flox/flox}$; named cVAChT mice henceforth) by crossing the Myh6-Cre mice (B6.FVB-Tg(Myh6-cre)2182Mds/J; Jackson Laboratory) with VAChT floxed mice [13] (Fig. 4.1a,b). The α-MHC (Myh6) promoter drives Cre expression exclusively in cardiomyocytes [30]. Although previous reports indicated that Cre is expressed mainly in ventricles in Myh6-Cre mice, Cre expression in the atria has been observed during development [31]. To confirm elimination of VAChT in cVAChT mice, we isolated adult cardiomyocytes and performed RT-PCR to detect VAChT transcripts (Fig. 4.2a). A band of 167 bp was detected in $VAChT^{flox/flox}$, but not in cVAChT mice. Sequencing analysis confirmed that this band represented the VAChT sequence. Moreover, immunoblot (Fig. 4.2b) and immunofluorescence analysis (Fig. 4.2c) confirmed elimination of VAChT in isolated ventricular cardiomyocytes from cVAChT mice. Moreover, whole-mount fluorescent IHC in the atria of control mice revealed that VAChT is also expressed in SA nodal cells, as co-localization was observed between VAChT and HCN4, a marker for the SA node (Fig. 4.2d). Importantly, VAChT staining in SA nodal cells was absent in cVAChT tissue indicating that VAChT was also deleted in these cells in cVAChT mice (Fig. 4.2d). Furthermore, VAChT expression in parasympathetic ganglia, recognized through their characteristic morphology, and in nerve terminals was not altered in cVAChT mice (Fig. 4.2d,e), indicating that we specifically eliminated non-neuronal VAChT in the heart. In support of these data, we used a reporter mouse line to demonstrate that Cre expression is absent in parasympathetic nerve terminals stained with an antibody against the high affinity choline transporter (CHT1; Fig. 4.1c). Additionally, expression of CHT1, the high affinity choline transporter was not affected in isolated

Figure 4.1 - Generation of cardiomyocyte–specific VAChT KO mice. (a) Schematic drawing of the VAChT alleles generated. Boxes represent the different exons of ChAT or VAChT. The position of the initiation codon (ATG) for VAChT and ChAT and the stop codon (Stop) of VAChT are indicated. P1, P2, and P3 indicate the primers used for PCR genotyping and the fragment sizes generated. LoxP sequence and some restriction enzymatic sites are represented. The VAChT gene is within the first intron of ChAT. **(b)** Representative genotyping results for mutant cVAChT ($VAChT^{flox/flox}$, Myh6-cre^{+}) mice (lanes 1 and 2) and control ($VAChT^{flox/flox-}$) mice (lanes 3 and 4 respectively). **(c)** YFP expression, which is regulated by Cre recombinase expression, in the ventricles (**c; top panel)** and intracardiac ganglia, specifically labeled with CHT1, in the right atrium **(c; bottom panel)** in Myh6-Cre^{+};$Rosa^{+}$ mice. The nuclei are labeled in blue using the TO-PRO 3 counterstain. The Myh6-Cre mice were used to generate cardiomyocyte-specific VAChT knockout (cVAChT) mice. Assessment of CHT1 **(d)** and ChAT **(e)** expression via immunofluorescence in isolated cardiomyocytes from cVAChT mice (scale bar=25 μm).

Figure 4.1 - Generation of cardiomyocyte–specific VAChT KO mice.

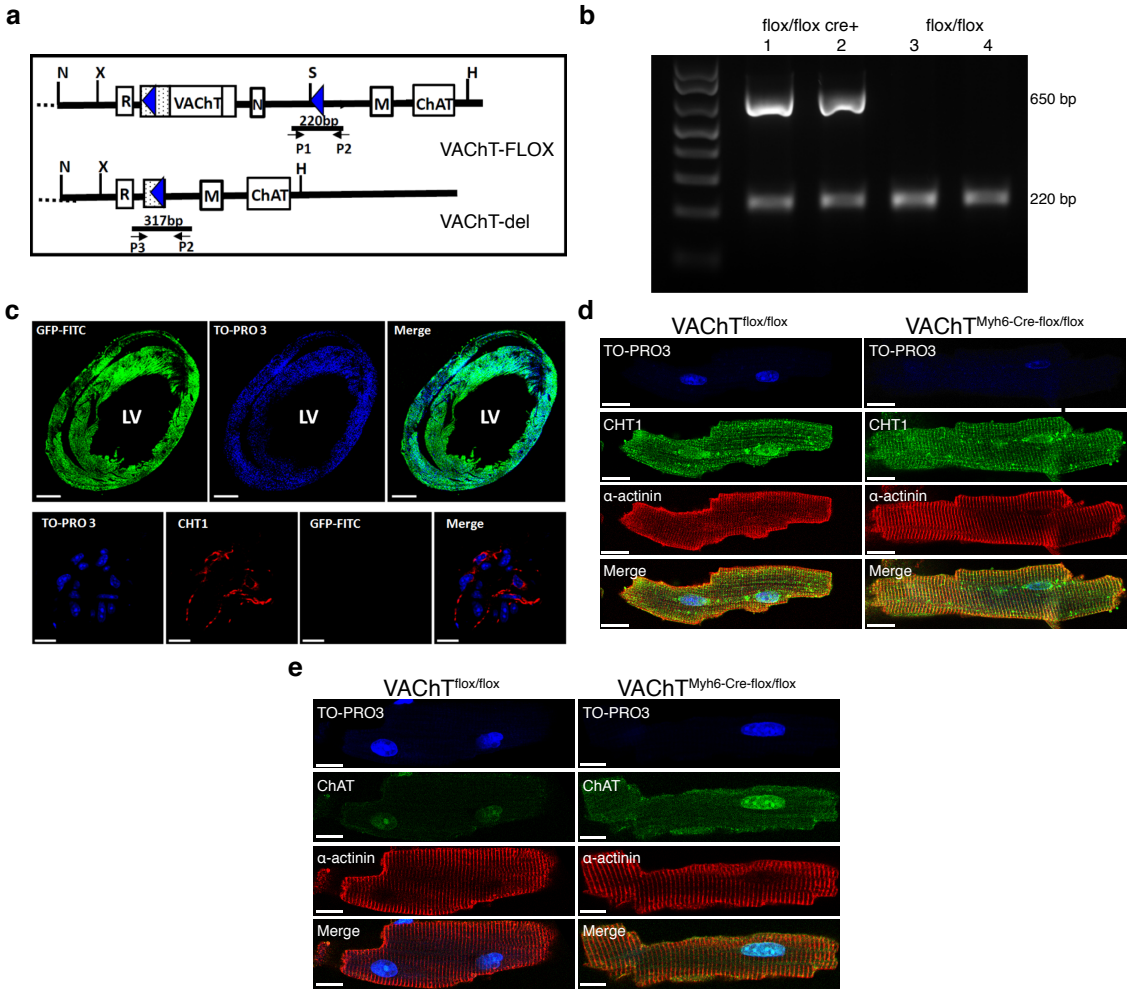
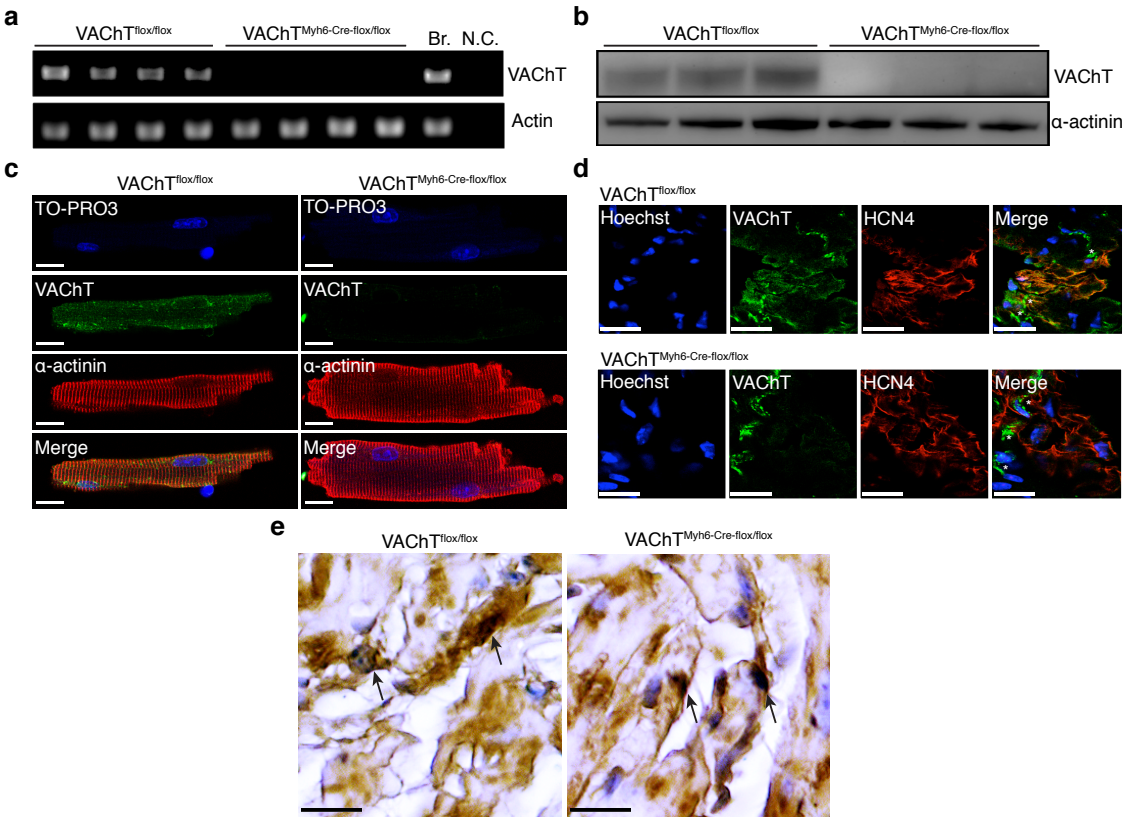


Figure 4.2 - Selective elimination of VAChT in cardiomyocytes of cVAChT mice. VAChT expression was determined by **(a)** PCR (Br=Brain, NC=Negative control), **(b)** immunoblotting and **(c)** immunofluorescence in adult cardiomyocytes. **(d)** Co-labeling for VAChT and HCN4 in whole-mount atrial tissue from control and cVAChT mice. **(e)** VAChT immunoreactivity in intracardiac parasympathetic ganglia from cVAChT mice (arrows indicate positive staining for parasympathetic ganglia).

Figure 4.2 - Selective elimination of VAChT in cardiomyocytes of cVAChT mice.



cardiomyocytes from cVAChT mice (Fig. 4.1d). However, cVAChT cardiomyocytes appeared to have greater levels of ChAT expression (Fig. 4.1e), an observation similar to that previously reported in VAChT KO mice, which reflects, in part, a rearrangement of the cholinergic gene *locus* following Cre-mediated recombination [23].

4.5.2 Genetic removal of VAChT from cardiomyocytes inhibits ACh release

Secretion of ACh from cultured neonatal cardiomyocytes was analyzed using a fluorometric assay to detect choline/ACh levels in culture media. The assay was performed in neonatal cardiomyocytes after 4 days in culture, to avoid potential contribution of parasympathetic neurons that could contaminate adult cardiomyocyte preparations. Using this method, we were able to confidently detect as low as 10 pmols/well for ACh (Fig. 4.3a). ACh release from control myocytes could be easily detected in the presence of pyridostigmine, a cholinesterase inhibitor used to preserve secreted neurotransmitter (Fig. 4.3b). In the presence of vesamicol, a specific inhibitor of the VAChT, ACh release from these cells was significantly diminished (Fig. 4.3b). In agreement with the pharmacological data, cVAChT cardiomyocytes showed no detectable ACh release (Fig. 4.4a).

We have confirmed these findings by performing HPLC with electrochemical detection of ACh in media from cultured cardiomyocytes. We were able to confidently detect up to 125 fmol of ACh on the column (Fig. 4.3c). ACh secretion was detectable from wild-type cardiomyocytes in the presence of pyridostigmine (Fig. 4.3d); however, ACh secretion from cells treated with vesamicol was significantly reduced (Fig. 4.3d). Furthermore, no ACh was detected in media from cVAChT cardiomyocytes using HPLC with electrochemical detection, whereas ACh could be detected in media from WT cardiomyocytes (Fig. 4.4b; Fig. 4.3e).

Figure 4.3 - Measurement of ACh release from neonatal cardiomyocytes. (a) Standard curve for measurement of ACh ranging from 10-50 pmols/well using a fluorometric choline/acetylcholine assay. **(b)** Measurement of ACh release from cultured, neonatal cardiomyocytes in the presence of pyridostigmine alone or pyridostigmine and vesamicol together. n=number of separate cell isolations. ***$p<0.001$ vs. control. **(c)** Standard curve for measurement of ACh ranging from 125-1000 pmols OTC using HPLC-ED. **(d)** Measurement of ACh release from wild-type neonatal cardiomyocytes using HPLC-ED in the presence of pyridostigmine alone or pyridostigmine and vesamicol together. n=number of separate cell isolations. ***$p<0.001$ vs. control. **(e)** Representative chromatograms of ACh release from control and cVAChT cardiomyocytes (blank and 500 fmol standard OTC are shown for comparison). **(f)** NO levels as an indirect measure of ACh release in cultured neonatal cardiomyocytes using DAF fluorescence. Cells were treated with either carbachol (Carb.) to activate muscarinic receptors and increase NO levels or pyridostigmine (PYR), which preserves secreted ACh that can increase NO production[12]. Cardiomyocytes were also co-treated with PYR and either vesamicol (VAChT inhibitor) or hemicholinium-3 (CHT1 inhibitor). n=number of cells examined from five separate cell isolations. Scale bar=25 μm. *$p<0.05$ vs. control and doubled-treated groups.

Figure 4.3 - Measurement of ACh release from neonatal cardiomyocytes.

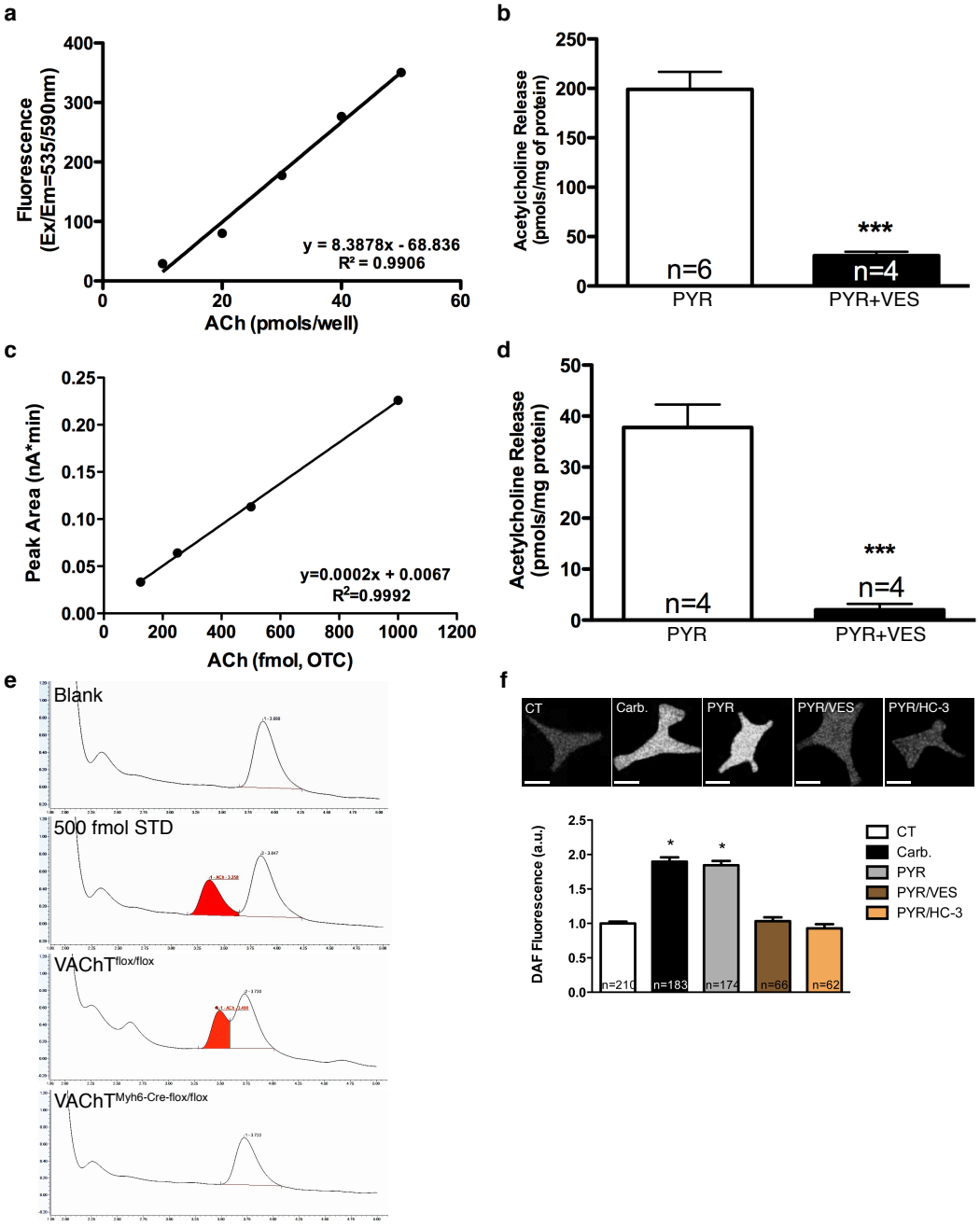
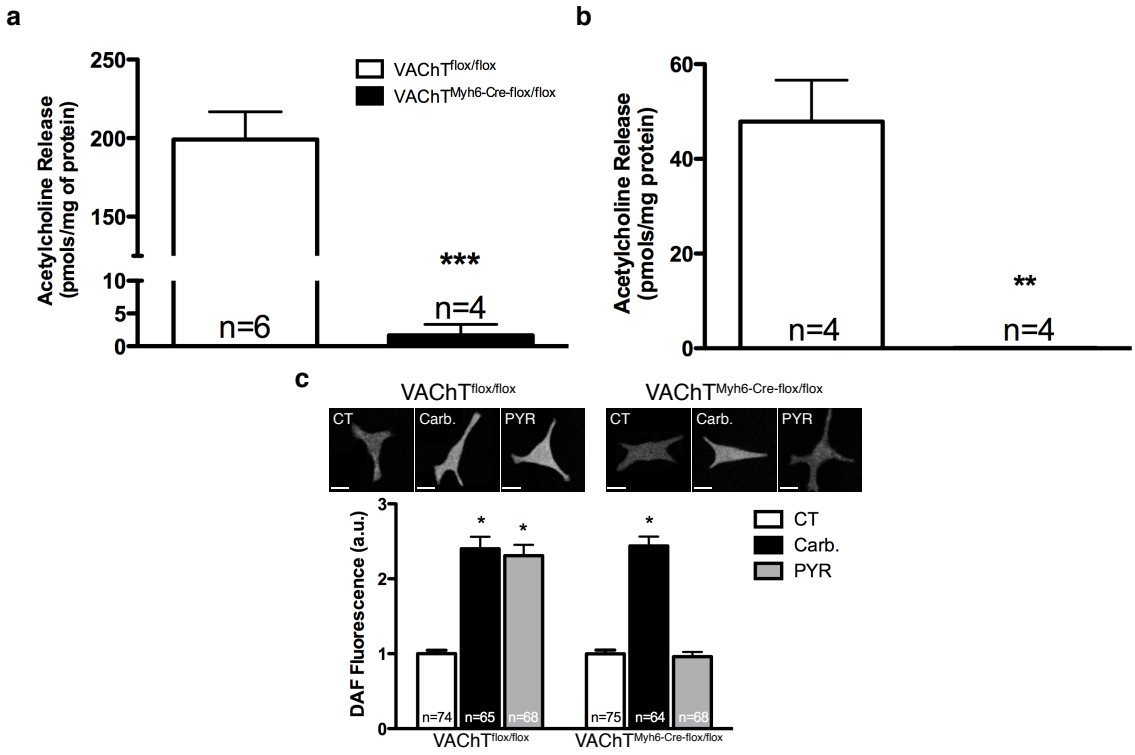


Figure 4.4 - Acetylcholine secretion is inhibited from cVAChT cardiomyocytes. (a) Acetylcholine release from neonatal cardiomyocytes isolated and cultured from control and cVAChT mice. n=number of separate cell isolations for each genotype. ***$p<0.001$ vs. control. **(b)** Acetylcholine release from control and cVAChT neonatal cardiomyocytes as detected through HPLC-Electrochemical Detection. n=number of separate cell isolations for each genotype. **$p<0.01$ vs. control. **(c)** Bioassay to measure ACh release in cultured neonatal cardiomyocytes using DAF fluorescence. Cells were treated with either carbachol (Carb.) to activate muscarinic receptors and increase NO levels or pyridostigmine (PYR), which preserves secreted ACh that can then activate muscarinic receptors and increase the production of NO(12). n=number of cells examined from five separate cell isolations for each genotype. Scale bar=25 μm. *$p<0.05$ vs. control. Data are represented as the mean ± SEM.

Figure 4.4 - Acetylcholine secretion is inhibited from cVAChT cardiomyocytes.



We further confirmed that secretion of ACh was inhibited in cVAChT neonatal myocyte cultures through the use of a bioassay with the fluorescent dye DAF-FM, as previously described [12]. DAF becomes fluorescent in the presence of nitric oxide (NO), which is produced by activation of muscarinic receptors by secreted ACh from cardiomyocytes. To validate this bioassay we used neonatal cardiomyocytes from C57BL/6 wild-type (WT) mice. We showed that DAF fluorescence was significantly increased in WT cardiomyocytes following treatment with carbachol (a muscarinic agonist; Fig. 4.3f). Pyridostigmine also augmented the fluorescent signal (Fig. 4.3f). Conversely, no fluorescence increase was observed in cardiomyocytes co-treated with pyridostigmine and either hemicholinium-3 (to inhibit the production of ACh) or vesamicol (Fig. 4.3f). These results indicate that this bioassay has enough sensitivity to detect changes in ACh release from WT cardiomyocytes. When cardiomyocytes from cVAChT mice were tested, DAF fluorescence was significantly increased in response to carbachol; however, no response was observed following treatment with the cholinesterase inhibitor pyridostigmine. In contrast, response for cardiomyocytes from littermate controls presented a robust response to carbachol and cholinesterase inhibition (Fig. 4.4c). Together, these results clearly establish using multiple methodologies that secretion of ACh from cardiomyocytes is impaired in cVAChT mice.

4.5.3 Inhibition of ACh secretion from cardiomyocytes alters heart activity

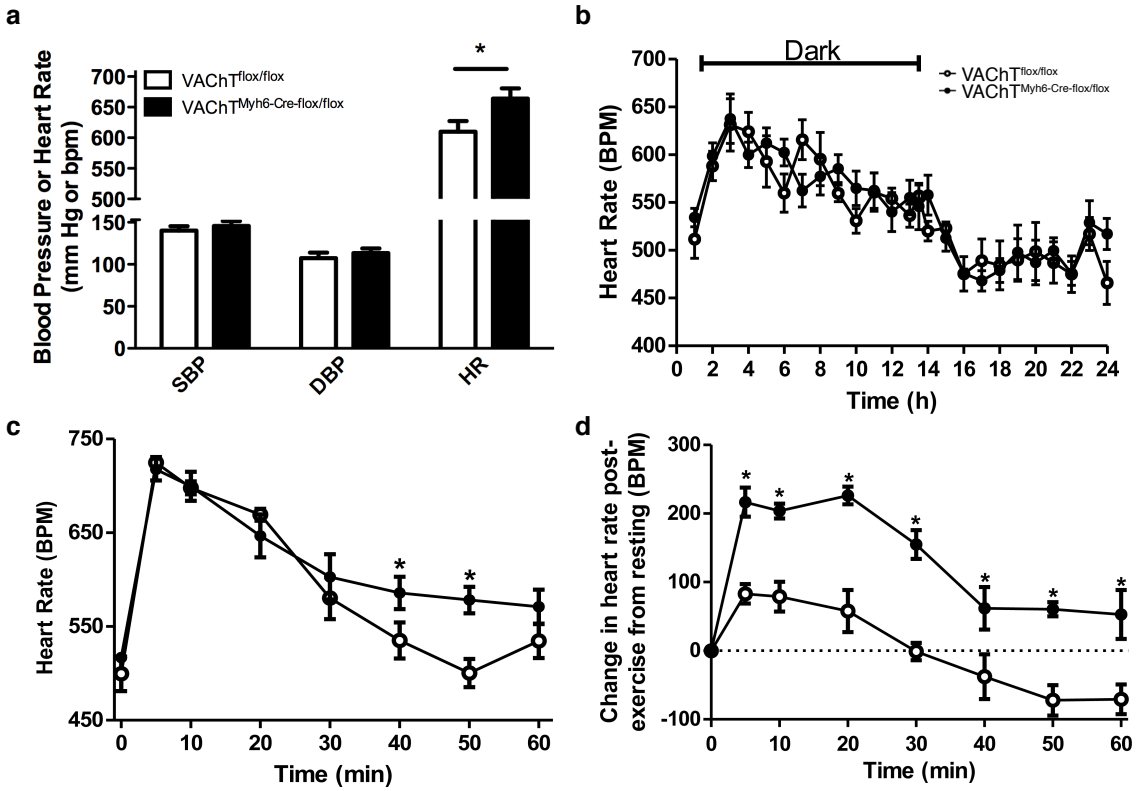
cVAChT mice did not present any gross abnormalities in appearance or body weight (29.1±4.1g in control vs. 30.8±4.9 g in cVAChT mice). Furthermore, these mice did not present alterations in several metabolic parameters, as measured using metabolic cages (Table 4.1), suggesting that the cardiomyocyte changes observed were not deleterious to mutant mice under control conditions. Accordingly, we did not observe changes in blood pressure as determined by tail-cuff (Fig. 4.5a), nor in basal heart rate as measured by radio telemetry (ECG, Fig. 4.5b). In contrast, cVAChT mice showed an increase in heart rate when analysed using the non-invasive tail-cuff system (Fig. 4.5a). This increase in heart rate was not due to the presence of Cre recombinase, as it was not observed in

Table 4.1 – Metabolic activity and inactivity parameters for VAChT$^{flox/flox}$ (n=6) and cVAChT (n=6) mice.

Parameter	Light 12 h		Dark 12 h	
	VAChT$^{flox/flox}$	***cVAChT***	***VAChT$^{flox/flox}$***	***cVAChT***
Food intake (g/12 h)	0.91 ± 0.92	0.92 ± 0.11	2.54 ± 0.35	2.44 ± 0.23
Water (ml/12 h)	0.73 ± 0.17	1.12 ± 0.21	2.44 ± 0.27	3.04 ± 0.22
VO_2 (ml/kg/h)	2390.1 ± 120.3	2489.8 ± 177.1	3082.5 ± 180.2	3242.0 ± 194.1
VCO_2 (ml/kg/h)	2239.3 ± 133.8	2378.1 ± 143.5	2948.7 ± 175.2	3123.4 ± 153.8
RER	0.94 ± 0.04	0.96 ± 0.02	0.96 ± 0.02	0.97 ± 0.02
Energy expenditure (kcal/h)	0.36 ± 0.01	0.38 ± 0.02	0.46 ± 0.02	0.50 ± 0.03
Total activity (counts/h)	1229.8 ± 55.7	1408.8 ± 86.3	3791.3 ± 443.4	4442.9 ± 597.1
Amb. activity (counts/h)	306.1 ± 32.4	428.3 ± 67.0	1531.2 ± 305.9	2024.0 ±342.5
Sleep time (min)	422.7 ± 10.0	404.4 ± 9.3	187.7 ± 10.8	199.0 ± 21.7
RER: Respiratory exchange rate; Amb. activity: Ambulatory activity				

Figure 4.5 - Analysis of heart rate in cVAChT mice. (a) Blood pressure and heart rate analysis in VAChT$^{flox/flox}$ and cVAChT mice using the CODA tail cuff system. **(b)** Heart rate over 24 hours in awake, free moving VAChT$^{flox/flox}$ and cVAChT mice in their home cage (n≥4 mice/genotype). **(c)** Heart rate response following gentle restraint and IP saline injection (n≥4 mice/genotype). **(d)** Heart rate recovery following acute, brief exercise in VAChT$^{flox/flox}$ and cVAChT mice (n≥4 mice/genotype). Data are represented as the mean ± SEM. *$p<0.05$ vs. control mice.

Figure 4.5 - Analysis of heart rate in cVAChT mice.



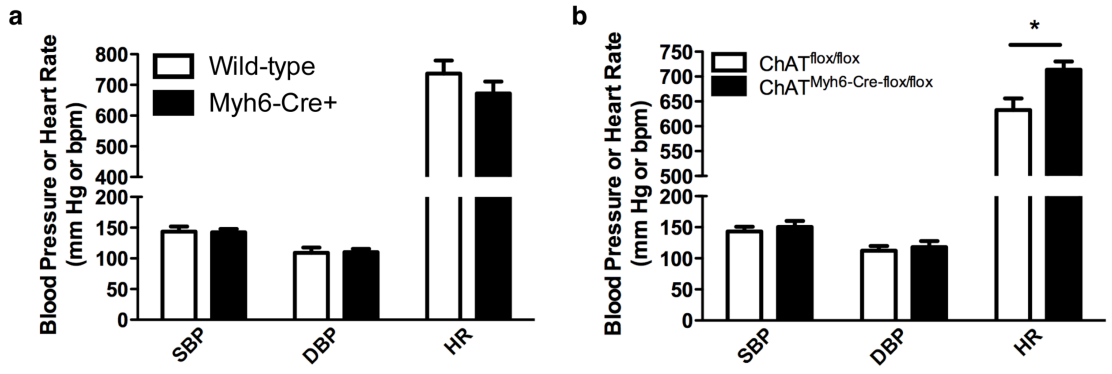
Myh6-cre mice were compared to wild-type mice under the same conditions (Fig. 4.6a). These results suggest a possible role for cardiomyocyte-derived ACh in the regulation of heart rate.

In order to further confirm that the increased heart rate observed in cVAChT mice via tail-cuff was due to inhibition of the non-neuronal cholinergic system in cardiomyocytes, we generated cardiomyocyte-specific ChAT knockout mice ($ChAT^{Myh6\text{-}Cre\text{-}flox/flox}$). This ChAT floxed line has been previously characterized and, when crossed to Cre lines, ChAT expression is abolished in a manner that parallels elimination of ACh [15, 32]. Tail-cuff analysis in these mice also revealed increased heart rate in mice lacking ACh production (Fig. 4.6b).

Tail-cuff measurements require physical restraint, which is stressful for mice, while ECG measurements are obtained under conditions similar to the home cage environment. Hence, we hypothesized that the difference in heart rate observed using these two different techniques might reflect an imbalance in cardiac regulation in mutant mice. Specifically, that cardiomyocyte-derived ACh could play a physiological role in boosting the parasympathetic signalling required for recovery of heart rate in response to stress. To test this hypothesis we used ECG telemetry to record heart rates immediately after mice were stressed by an intraperitoneal injection of saline. We observed that heart rate recovery of cVAChT mice to baseline levels was slower when compared to control $VAChT^{flox/flox}$ mice (Fig. 4.5c). These data suggest that, following increased sympathetic demand due to stress, regulation of heart function by the parasympathetic system relies, at least in part, on ACh secreted from cardiomyocytes. To further test this possibility, cVAChT and littermate controls implanted with ECG telemeters were submitted to an acute, low intensity, treadmill exercise test. Immediately following this mild exercise routine, heart rates in control and cVAChT mice were recorded in their home cage. The results demonstrate that this exercise routine led to a significantly greater increase in heart rate in cVAChT mice as compared to control mice (Fig. 4.5d). Moreover, heart rate recovery to pre-exercise levels took significantly longer in cVAChT mice when compared to control mice (Fig. 4.5d). These results suggest that cardiomyocyte-derived

Figure 4.6 - Tail-cuff analysis using the non-invasive CODA system. (a) Blood pressure and heart rate analysis in WT and Myh6-Cre+ mice (n≥5 mice/genotype). **(b)** Blood pressure and heart rate analysis in $ChAT^{flox/flox}$ and $ChAT^{Myh6\text{-}Cre\text{-}flox/flox}$ mice using the CODA tail cuff system, n≥8 mice/genotype. Data are represented as mean ± SEM.

Figure 4.6 - Tail-cuff analysis using the non-invasive CODA system.



ACh may normally offset sympathetic activity, helping the heart to respond to increased sympathetic demand.

4.5.4 cVAChT mice display cardiac hypertrophy and molecular remodeling

The results above suggest that control of heart rate, particularly under stress, is affected in cVAChT mice potentially due to decreased cholinergic signalling. Previous experiments indicated that *in vitro* inhibition of either VAChT activity or ACh synthesis increased hypertrophy in cultured neonatal cardiomyocytes treated with sympathetic agonists [12]. In order to determine whether cardiomyocyte-secreted ACh plays a role in cardiac remodelling *in vivo*, we analyzed heart weight in 3 month-old mutant mice. As shown in Fig. 4.7a, cVAChT mice showed increased heart size. Cardiac hypertrophy was due to remodelling of cVAChT myocytes, which exhibited a significant increase in surface area when measured both *in vitro* (Fig. 4.7b) and *in situ* (Fig. 4.7c). Importantly, the presence of Cre recombinase alone did not lead to either cardiac or cardiomyocyte hypertrophy (Fig. 4.8a, b).

To further test whether the observed hypertrophic response was indeed dependent on VAChT expression and activity, we cultured wild-type neonatal cardiomyocytes and treated them with a pharmacological inhibitor of VAChT activity (vesamicol), or with siRNA against VAChT (Fig. 4.8c). VAChT knockdown was confirmed through qPCR and immunoblotting, which revealed an 80% and 60% decrease in VAChT mRNA and protein levels, respectively. Both of these treatments led to a significant increase in cardiomyocyte size after 48h in culture. Furthermore, cardiomyocytes treated with an inhibitor of CHT1 (hemicholinium-3), which blocks ACh synthesis, presented similar hypertrophy (Fig. 4.8c), thus confirming that the hypertrophic response was due to inhibition of cholinergic signalling at the level of the myocytes.

Cardiac hypertrophy is associated with increased expression of fetal program genes, which contribute to cardiac remodelling [33]. Expression of the markers of cardiac

Figure 4.7 - Cardiac hypertrophy in cVAChT mice. (a) Heart weight normalized to tibia length in $VAChT^{flox/flox}$ and cVAChT mice (n=number of mice). **(b)** Surface area of isolated cardiomyocytes (scale bar = 25 μm, n=number of mice/genotype, at least 75 cells were analyzed/genotype). **(c)** Surface area of cardiomyocytes *in situ* (scale bar = 25 μm, n=number of mice/genotype, at least 70 cells were analyzed/genotype). Data are represented as the mean ± SEM. *$p<0.05$ vs. control.

Figure 4.7 - Cardiac hypertrophy in cVAChT mice.

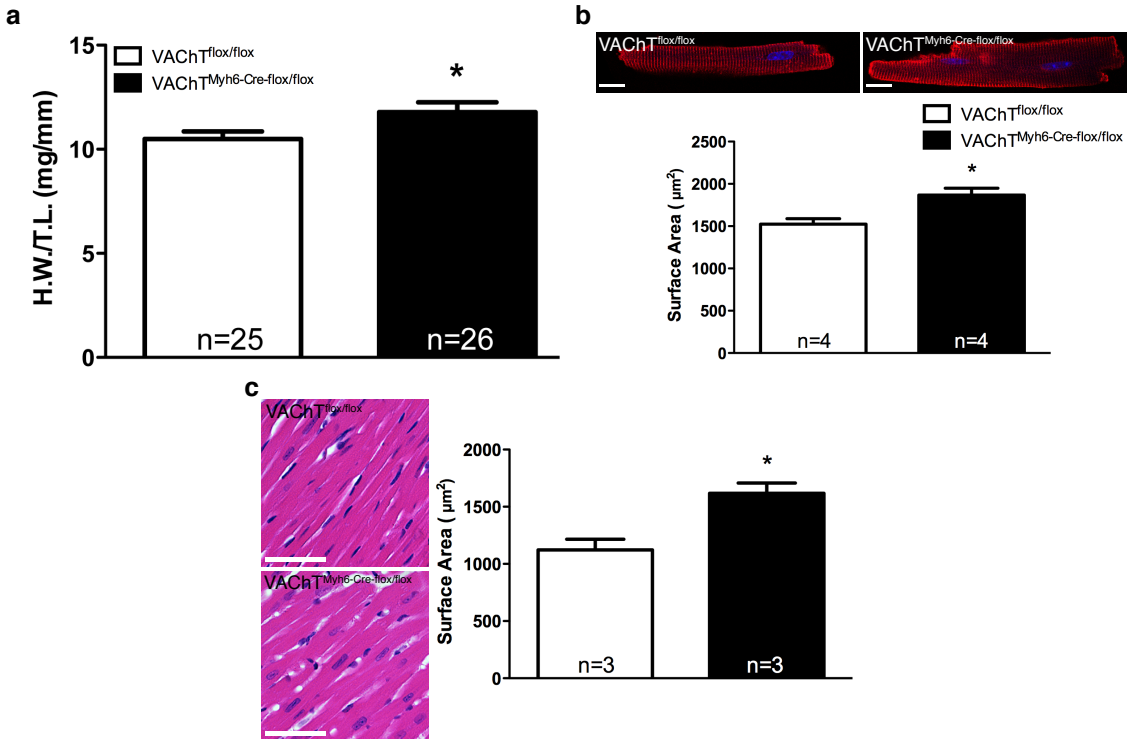
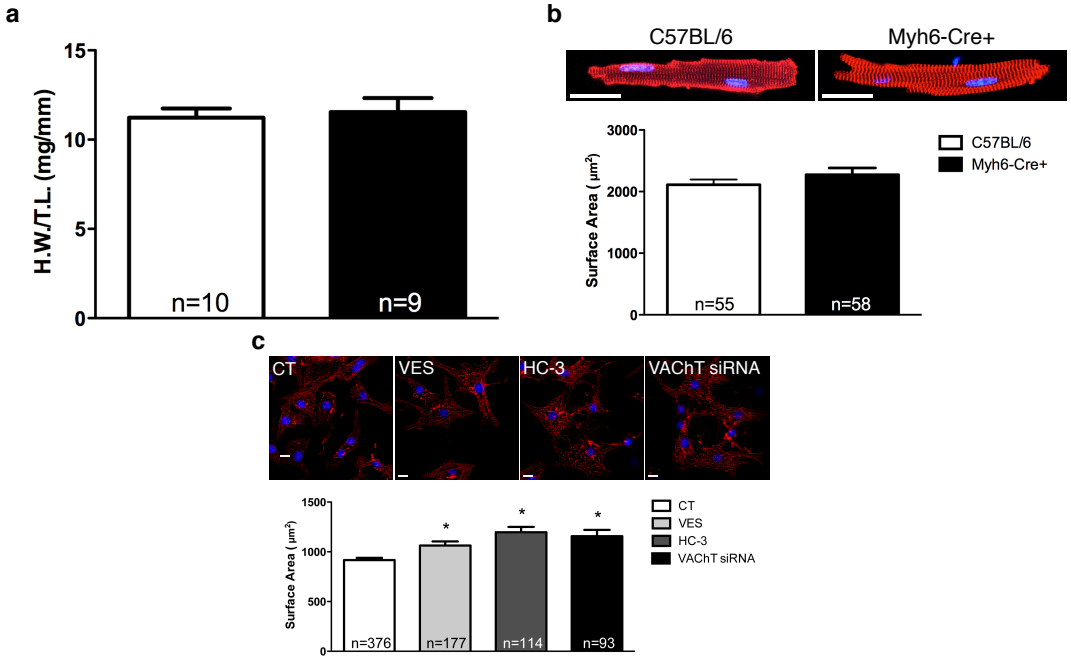


Figure 4.8 - Measurement of hypertrophy in mice expressing Cre recombinase or following inhibition of VAChT *in vitro*. (a) Heart weight normalized to tibia length in WT and Myh6-Cre+ mice (n=number of mice). **(b)** Surface area of isolated cardiomyocytes in WT and Myh6-Cre+ mice (n=number of cells from 3 separate isolations). Scale bar=25 μm. **(c)** Surface area of cultured neonatal cardiomyocytes following treatment with the VAChT inhibitor vesamicol (VES), the CHT1 inhibitor hemicholinium-3 (HC-3) or VAChT knockdown using siRNA (n=number of cells). VAChT knockdown was confirmed through qPCR and immunoblotting, which revealed an 80% and 60% decrease in VAChT levels, respectively. All images are representative of, at least, three independent experiments in which multiple cells were examined. *$p<0.05$ vs. control.

Figure 4.8 - Measurement of hypertrophy in mice expressing Cre recombinase or following inhibition of VAChT *in vitro*.



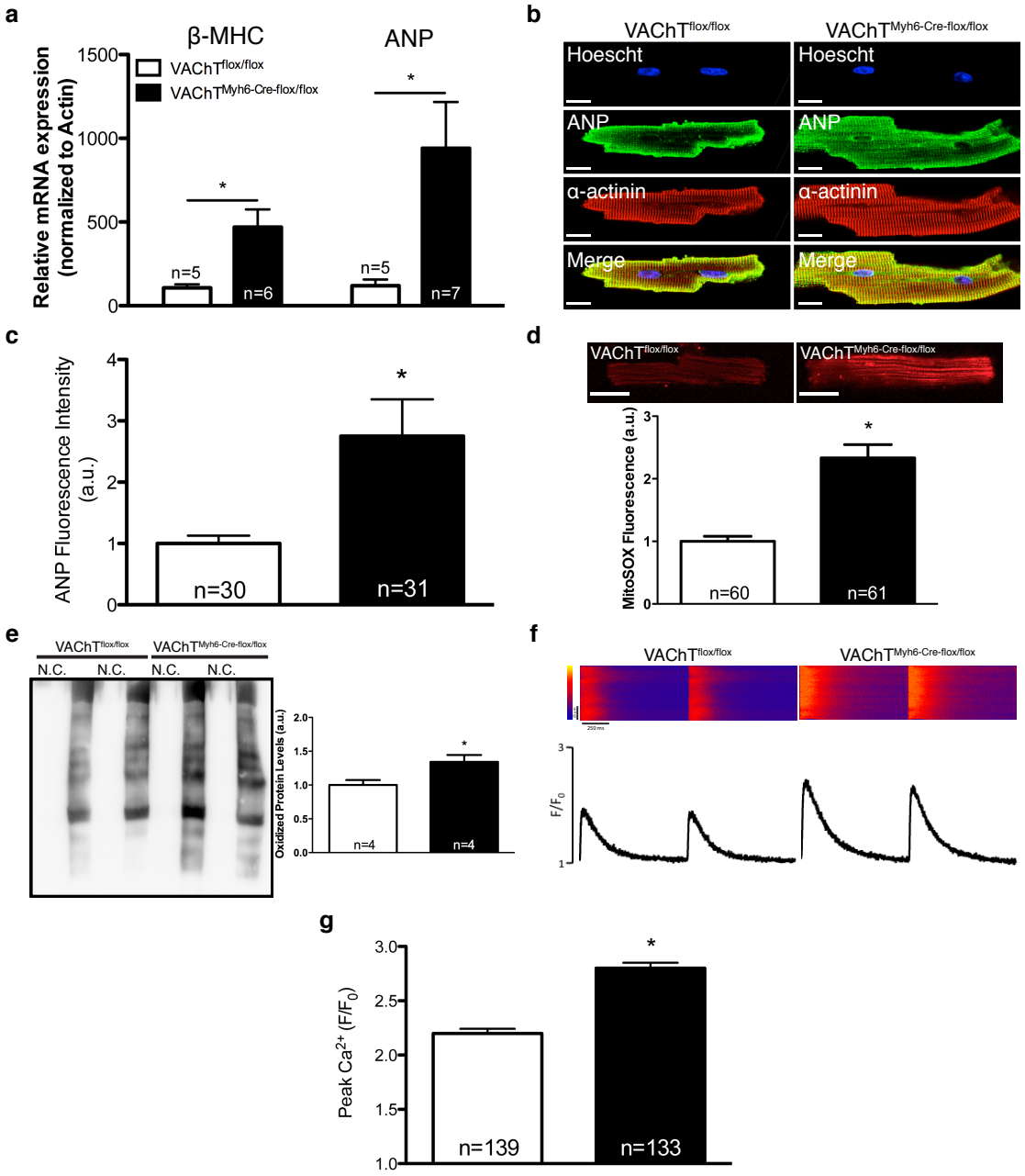
remodelling and stress, β-myosin heavy chain (β-MHC) and atrial natriuretic peptide (ANP), was increased several fold in cardiomyocytes from cVAChT mice (Fig. 4.9a). Immunostaining in isolated cardiomyocytes revealed also increased ANP protein levels (Fig. 4.9b,c), compatible with cardiomyocyte remodelling. Cardiomyocyte remodelling and reactivation of the fetal gene program are usually associated with increased cellular stress [34]. Furthermore, hypertrophy induced through hyperadrenergic signalling has been coupled to increased ROS levels [35]. If removal of VAChT from cardiomyocytes leads to imbalanced autonomic control of the heart, it is possible that mutant cardiomyocytes are under considerably more stress than control cardiomyocytes. We tested this possibility and found that cardiomyocytes isolated from cVAChT mice showed increased levels of ROS (as determined via mitochondrial superoxide levels; Fig. 4.9d), suggesting that myocytes lacking the intrinsic cholinergic system display increased levels of stress. Furthermore, we determined that increased ROS levels in myocytes led to an increase in overall protein oxidation in the whole heart in cVAChT mice (Fig. 4.9e), as measured using the OxyBlot protein oxidation kit, which can detect the levels of oxidized proteins.

Increased levels of mitochondrial ROS have previously been shown to increase $[Ca^{2+}]_i$ in arterial myocytes, especially following induction of hypoxic stress [36]. Therefore, we sought to determine whether the increased levels of ROS observed in cVAChT myocytes were accompanied with changes in calcium transients in ventricular cardiomyocytes. In agreement with this possibility, we detected an alteration in calcium handling in cardiomyocytes obtained from mutant mice with an increase in peak calcium (Ca^{2+}) transients (Fig. 4.9f,g). These results suggest that elimination of VAChT in cardiomyocytes leads to remodelling of cardiomyocytes, with overactivation of Ca^{2+} transients potentially altering cardiac function.

It is likely that inhibition of the non-neuronal cholinergic system leads to imbalanced regulation of cardiac activity. As such, we analyzed whether components involved in sympathetic signalling and thus, regulation of cardiomyocyte function, were altered in cVAChT mice. G-protein coupled receptor kinases (GRKs) have been shown to play

Figure 4.9 - Cellular stress in cVAChT cardiomyocytes. (a) Expression of the cardiac stress markers, β-MHC and ANP, in control and cVAChT cardiomyocytes (n=number of mice). **(b, c)** Immunostaining for ANP in adult cardiomyocytes from control and cVAChT mice (scale bar=25 μm). **(d)** Measurement of ROS levels in isolated cardiomyocytes loaded with the MitoSOX superoxide indicator (scale bar=25 μm, n=number of cells; at least 3 mice/genotype). **(e)** Measurement of oxidized protein levels in whole hearts from control and cVAChT mice (n=number of mice). **(f, g)** Assessment of calcium transients in isolated ventricular myocytes from $VAChT^{flox/flox}$ and cVAChT mice. **(f)** Representative recordings of line-scan profile of Ca^{2+} transients in control and cVAChT myocytes **(g)** Summary of peak Ca^{2+} (n=number of cardiomyocytes; cells isolated from at least 3 mice/genotype). Data are represented as the mean ± SEM. *$p<0.05$ vs. control.

Figure 4.9 - Cellular stress in cVAChT cardiomyocytes.



important roles in cardiac hypertrophy in response to increased sympathetic activation [37, 38]. Therefore, we examined the expression of GRK2 and GRK5, the two most predominantly expressed GRKs in cardiac tissue. Although GRK2 did not show any changes in expression, mRNA expression of GRK5 was significantly increased in cVAChT myocytes (Fig. 4.10a). Moreover, immunostaining analysis showed that subcellular localization of GRK5 was altered in cVAChT cardiomyocytes, as mutated cells showed increased nuclear localization for GRK5 as compared to control cardiomyocytes (Fig. 4.10b,c).

4.5.5 Cardiomyocyte remodeling in cVAChT mice affects LV function

Ventricular cardiomyocytes from cVAChT mice display remodelling and hypertrophy; therefore, we tested whether these alterations affected left ventricular contractility. Invasive hemodynamic assessments were performed on anesthetised control and cVAChT mice. As shown in Table 4.2, the hemodynamic parameters were similar in both genotypes under baseline conditions; however, the LV hemodynamics were altered in cVAChT mice following a bolus dose of isoproterenol, used to increase cardiac contractility. Interestingly, although heart rate was similar in both genotypes under baseline, the cVAChT mice showed a significant decrease in heart rate following ISO treatment. Furthermore, the maximum rate of LV pressure rise (peak $+dP/dt_{max}$) was significantly decreased and the maximum rate of LV pressure fall (peak $-dP/dt_{min}$) showed a tendency for decrease in cVAChT mice ($p=0.0508$). In addition, the contractility index of cVAChT mice was not different from control counterparts under baseline; however, there was a significant reduction in the contractility index following ISO treatment in cVAChT mice. These results suggest compromised left ventricular function in the mutant mice, especially under increased cardiac demand, as induced through treatment with ISO.

Figure 4.10 - Cardiac remodeling in cVAChT cardiomyocytes. (a) mRNA expression of GRK5 in isolated cells from control and cVAChT mice (n=number of mice). **(b, c)** Immunostaining for GRK5 in isolated myocytes (scale bar=25 μm). Data are represented as the mean ± SEM. *$p<0.05$ vs. control.

Figure 4.10 - Cardiac remodelling in cVAChT cardiomyocytes.

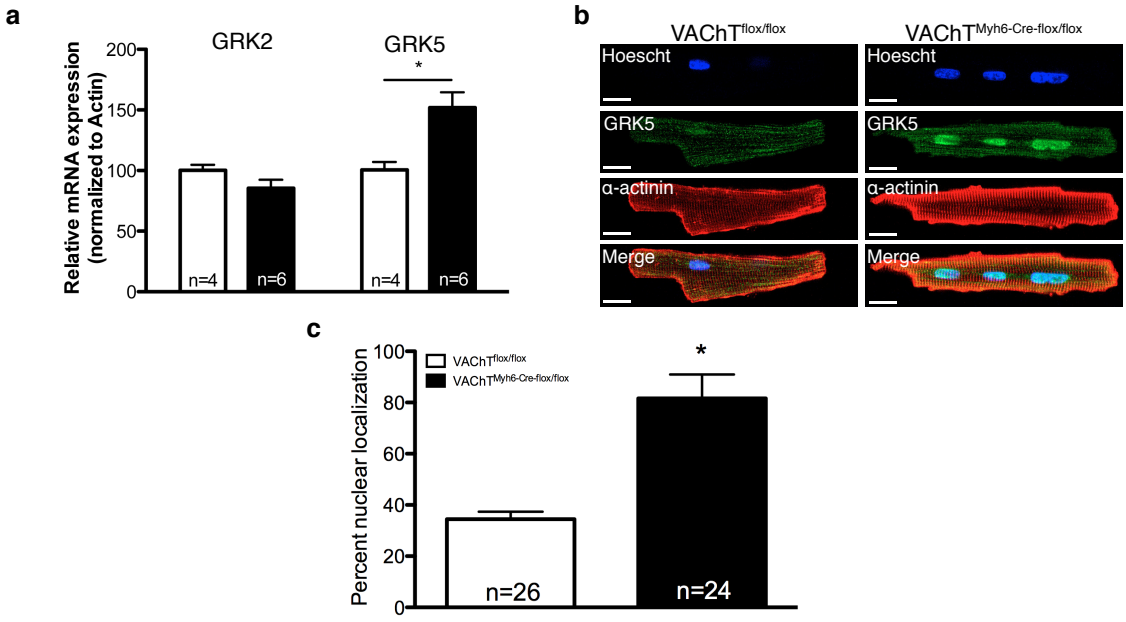


Table 4.2 – Hemodynamic parameters for control (n=9) and cVAChT (n=9) mice under baseline and following isoproterenol stimulation.

Parameter	Baseline		Isoproterenol	
	$VAChT^{flox/flox}$	$VAChT^{Myh6\text{-}cre\text{-}flox/flox}$	$VAChT^{flox/flox}$	$VAChT^{Myh6\text{-}cre\text{-}flox/flox}$
HR (bpm)	289.9 ± 24.7	267.4 ± 16.7	629.2 ± 11.2	566.1 ± 15.7*
LVSP (mm Hg)	103.3 ± 6.5	101.8 ± 4.0	97.2 ± 7.4	92.1 ± 5.6
LVEDP (mm Hg)	7.0 ± 2.4	12.5 ± 3.3	-0.3 ± 1.5	2.3 ± 2.0
$+dP/dT_{max}$ (mmHg/s)	7699 ± 809	6999 ± 720	16819 ± 1766	12432 ± 861*
$-dP/dT_{min}$ (mmHg/s)	-7370 ± 395	-6752 ± 518	-9770 ± 1019	-7413 ± 212‡
Contractility index (s^{-1})	154.8 ± 8.1	148.9 ± 13.9	347.5 ± 14.1	280.1 ± 15.6*

HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; $+dP/dT_{max}$, maximum first derivative of the change in left ventricle pressure; $-dP/dT_{min}$, minimum first derivative of the change in left ventricle pressure.
*$p<0.05$, ‡$p=0.0508$. Values are mean ± SEM.

4.6 Discussion

Our experiments reveal a novel and unexpected way by which cardiovascular function is regulated. These data indicate that cardiomyocytes can secrete significant amounts of ACh *in vivo* via a VAChT-dependent mechanism, similar to that observed in nerve terminals [39]. cVAChT mice seem to lack the capacity to sustain normal levels of cholinergic signalling, implicating non-neuronal ACh as part of a physiological system that controls cardiac function.

Lack of cardiomyocyte-secreted ACh leads to altered heart rate regulation under stress. The SA node controls heart rate; therefore, the alteration in heart rate we observed in cVAChT mice is likely related to the altered expression of VAChT in SA node myocytes observed in these mutants. Importantly, measurements of heart rate in the absence of stress indicate that regulation of basal activity does not seem to depend on this cardiomyocyte cholinergic system. Hence, contribution of cardiomyocyte-derived ACh is revealed only when there is increased demand on the parasympathetic system, such as following exercise. Importantly, an inverse relationship between decreased serum AChE levels and delayed heart rate recovery following exercise has previously been reported in healthy human subjects [40]. This previous work provides further support for the importance of peripheral ACh levels, including cardiomyocyte-derived ACh, in regulating cardiac function.

It is likely that non-neuronal ACh, secreted from cardiomyocytes, functions through similar second messenger systems as those activated when neuronal ACh binds to muscarinic receptors. Future electrophysiological studies will determine the mechanisms leading to alterations in chronotropic responses observed following inhibition of non-neuronal cholinergic signalling in the specialized cardiomyocytes of the SA node.

It is well established that increased sympathetic signalling can lead to cardiac remodelling and increased cardiomyocyte stress in the working myocardium [41]. Indeed,

even though cVAChT mice presented normal regulation of heart rate under non-stressful conditions, changes in heart rate, due to lack of cardiomyocyte-derived ACh, may cause long-term changes in heart function. It appears that lack of the intrinsic cholinergic system in cardiomyocytes leads to an increase in basal stress. This may play an important role in the regulation of cardiac function because it has previously been reported that increased stress can lead to an upregulation of AChE transcription in the brain [42]. It will be important to determine whether cVAChT mice display altered AChE transcription in the heart, which may then serve to further exacerbate the stress response by increasing degradation of neuronal ACh released at the level of the heart.

Lack of cardiomyocyte-derived ACh was associated to ventricular cardiomyocyte hypertrophy, molecular remodelling and increased oxidative stress, which argues for a widespread effect of cardiomyocyte-secreted ACh. These molecular changes were accompanied by alterations in left ventricular function and cardiac contractility under increased demand due to treatment with ISO. The observed hypertrophy may be related to the increased expression, as well as altered subcellular localization, of GRK5 observed in cVAChT cardiomyocytes. It has been reported that GRK5 overexpression leads to hypertrophy, which is dependent on its nuclear function [37]. Conversely, GRK5 null mice display a delay in hypertrophy following transverse aortic restriction [43]. Further studies will be required to elucidate potential GRK5-specific pathways affected in cVAChT myocytes that can lead to remodelling and hypertrophy. In addition, it will be important to determine whether the transcriptional activation of hypertrophic genes, including GRK5, are regulated by miRNAs as it has previously been shown that overexpression of the miR-212/132 family of miRNAs leads to pathological cardiac hypertrophy [44]. It will be interesting to determine whether lack of cardiomyocyte-derived ACh leads to hypertrophy in a miRNA-dependent mechanism.

Interestingly, the increase in calcium transients observed in isolated cVAChT cardiomyocytes would suggest a significant increase in inotropic response, *in vivo*. However, the Millar catheter experiments suggest that LV hemodynamics, specifically contractility and relaxation, are both significantly decreased following hyperadrenergic

stimulation using a bolus dose of isoproterenol. These apparently contradictory results might be due to a decrease in myofilament calcium sensitivity at the level of troponin. In addition, it is also possible that the increase in mitochondria superoxide levels leads to a deficiency in ATP production in cVAChT cardiomyocytes, which might result in inhibition of calcium uptake into the SR.

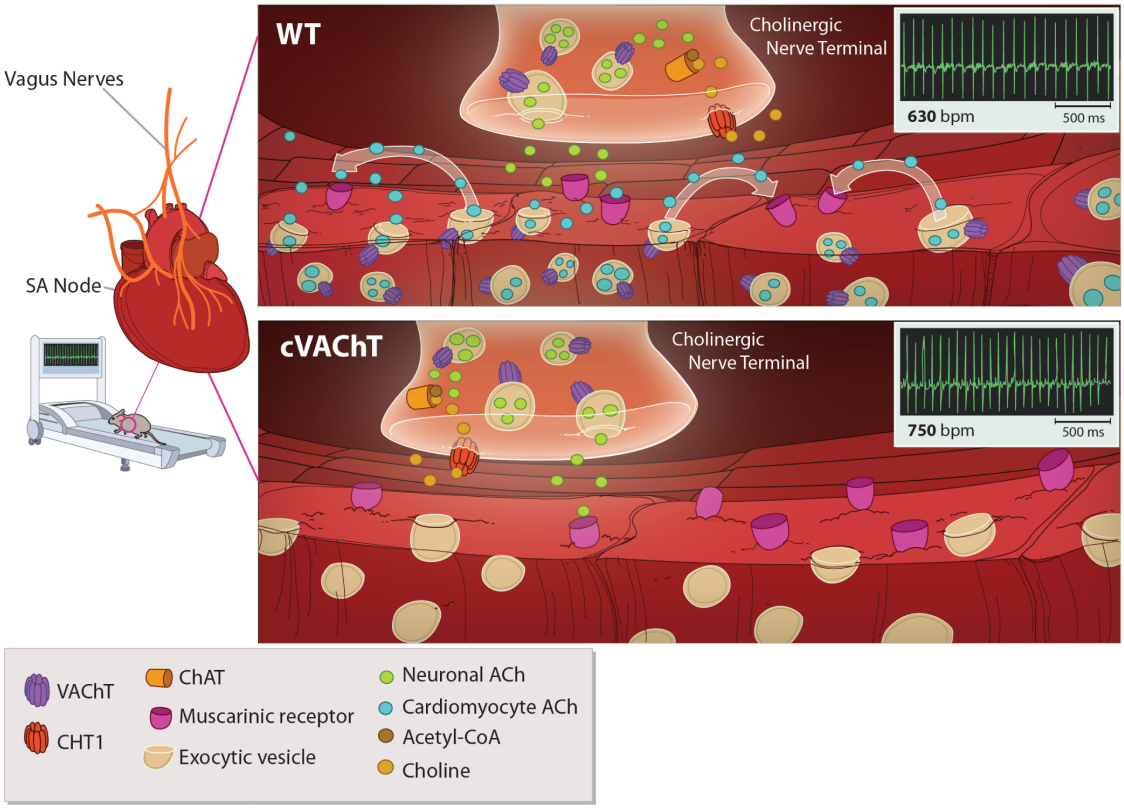
Although the detailed mechanisms that trigger the release of ACh from cardiomyocytes are not clear at the moment, a clear possibility might involve sympathetic signalling. Previous experiments *in vitro* suggest that adrenergic stimulation can induce the expression of cholinergic genes in cardiomyocytes [12]. Whether there are reciprocal interactions between sympathetic activation and cardiomyocyte-derived ACh is unknown. Interestingly, previous experiments have demonstrated that proteins involved with exocytic release of neurotransmitters, or their homologues, are expressed in vesicular compartments in cardiomyocytes [45]. Hence, secretory vesicle compartments in cardiomyocytes are likely to be the source of ACh secretion (Fig. 4.11). It should be noted we have previously established that VAChT is present in recycling vesicles in cardiomyocytes [12]. Our observation that the cholinergic cardiomyocyte machinery seems to control heart rate after stress and exercise suggests that cardiomyocyte-derived ACh secretion may be regulated by sympathetic activity.

Interestingly, activation of the cholinergic anti-inflammatory pathway has been shown to play an important role in preventing the release of pro-inflammatory cytokines from circulating macrophages and thereby attenuating the effects of infection or injury [46]. Immune cells can serve as a non-neuronal source of ACh and its levels appear to be altered in a miR-132 dependent mechanism involving targeting of AChE mRNA [47]. Although the activity of the cholinergic anti-inflammatory reflex has not been measured, it is expected that its function will be unaltered due to the specificity of the Cre line used.

Recently, non-neuronal secretion of ACh in a VAChT-dependent manner was demonstrated from pancreatic α cells, a process which regulates insulin secretion in humans [6]. Here, we show that secretion of ACh from cardiomyocytes also has

Figure 4.11 - Non-neuronal release of acetylcholine from cardiomyocytes. Wild-type cardiomyocytes secrete acetylcholine in response to increased physiological stress (e.g. exercise) in a VAChT-dependent manner to regulate heart rate. This response is blunted in mice lacking VAChT specifically in cardiomyocytes due to the lack of non-neuronal ACh release.

Figure 4.11 - Non-neuronal release of acetylcholine from cardiomyocytes.



physiological relevance as this non-neuronal cholinergic system plays a previously unsuspected role in regulating heart rate as well as remodelling. Based on these results, it is necessary to re-evaluate how the sympathetic-parasympathetic systems control heart activity to include this novel form of autocrine/paracrine communication from cardiomyocytes. We propose that secretion of ACh from cardiomyocytes enhances cholinergic signalling from parasympathetic neurons required to regulate cardiomyocyte activity (Fig. 4.11). Cardiomyocyte-derived ACh augments extracellular levels of this neurotransmitter in synaptic junctions between parasympathetic terminals and cardiomyocytes during high demand; for example, in response to increased sympathetic activation due to physiological stress. This novel mode of communication may serve to offset constant sympathetic signalling avoiding increased cardiomyocyte stress and hypertrophic responses.

4.7 Acknowledgments

We thank Dr. R. Jane Rylett for kindly providing the CHT1 antibody and Drs. Dale Laird and Qingping Feng (University of Western Ontario) for their insightful comments regarding the manuscript. This work was supported by the Heart and Stroke Foundation of Ontario (Grant NA6656 and G-13-0002843), Canadian Institutes for Health Research (Grant MOP-82756 and MOP-89919), Canadian Foundation for Innovation, the Ontario Research Fund and the National Institutes for Health (Grant R03TW008425 from the Fogarty International Center to S.G.). A..R is supported by an Ontario Graduate Scholarship. R.G. was supported by a New Investigator Award from the Heart and Stroke Foundation of Canada.

4.8 References

[1] Levy MN. Neural control of cardiac function. Bailliere's clinical neurology. 1997;6:227-44.

[2] Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. The Journal of clinical investigation. 2010;120:408-21.

[3] Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, et al. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol. 2010;30:1746-56.

[4] Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovascular research. 2004;62:112-21.

[5] Kawano H, Okada R, Yano K. Histological study on the distribution of autonomic nerves in the human heart. Heart and vessels. 2003;18:32-9.

[6] Rodriguez-Diaz R, Dando R, Jacques-Silva MC, Fachado A, Molina J, Abdulreda MH, et al. Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans. Nature medicine. 2011;17:888-92.

[7] Fujii T, Kawashima K. An independent non-neuronal cholinergic system in lymphocytes. Japanese journal of pharmacology. 2001;85:11-5.

[8] Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science. 2011;334:98-101.

[9] Wessler I, Kirkpatrick CJ, Racke K. Non-neuronal acetylcholine, a locally acting molecule, widely distributed in biological systems: expression and function in humans. Pharmacology & therapeutics. 1998;77:59-79.

[10] Kawashima K, Fujii T. Extraneuronal cholinergic system in lymphocytes. Pharmacology & therapeutics. 2000;86:29-48.

[11] Rana OR, Schauerte P, Kluttig R, Schroder JW, Koenen RR, Weber C, et al. Acetylcholine as an age-dependent non-neuronal source in the heart. Autonomic neuroscience : basic & clinical. 2010;156:82-9.

[12] Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, de Morais Gomes ER, et al. Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. Journal of molecular and cellular cardiology. 2012;53:206-16.

[13] Guzman MS, De Jaeger X, Raulic S, Souza IA, Li AX, Schmid S, et al. Elimination of the vesicular acetylcholine transporter in the striatum reveals regulation of behaviour by cholinergic-glutamatergic co-transmission. PLoS biology. 2011;9:e1001194.

[14] Martins-Silva C, De Jaeger X, Guzman MS, Lima RD, Santos MS, Kushmerick C, et al. Novel strains of mice deficient for the vesicular acetylcholine transporter: insights on transcriptional regulation and control of locomotor behavior. PloS one. 2011;6:e17611.

[15] Misgeld T, Burgess RW, Lewis RM, Cunningham JM, Lichtman JW, Sanes JR. Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. Neuron. 2002;36:635-48.

[16] Guatimosim S, Amaya MJ, Guerra MT, Aguiar CJ, Goes AM, Gomez-Viquez NL, et al. Nuclear Ca2+ regulates cardiomyocyte function. Cell calcium. 2008;44:230-42.

[17] Roy A, Lara A, Guimaraes D, Pires R, Gomes ER, Carter DE, et al. An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with decreased cholinergic neurotransmission. PloS one. 2012;7:e39997.

[18] Ribeiro FM, Alves-Silva J, Volknandt W, Martins-Silva C, Mahmud H, Wilhelm A, et al. The hemicholinium-3 sensitive high affinity choline transporter is internalized by clathrin-mediated endocytosis and is present in endosomes and synaptic vesicles. Journal of neurochemistry. 2003;87:136-46.

[19] Lau JK, Brown KC, Thornhill BA, Crabtree CM, Dom AM, Witte TR, et al. Inhibition of cholinergic signaling causes apoptosis in human bronchioalveolar carcinoma. Cancer research. 2013;73:1328-39.

[20] Friedman A, Kaufer D, Shemer J, Hendler I, Soreq H, Tur-Kaspa I. Pyridostigmine brain penetration under stress enhances neuronal excitability and induces early immediate transcriptional response. Nature medicine. 1996;2:1382-5.

[21] Kaufer D, Friedman A, Seidman S, Soreq H. Acute stress facilitates long-lasting changes in cholinergic gene expression. Nature. 1998;393:373-7.

[22] Lorke DE, Hasan MY, Nurulain SM, Shafiullah M, Kuca K, Petroianu GA. Pretreatment for acute exposure to diisopropylfluorophosphate: in vivo efficacy of various acetylcholinesterase inhibitors. Journal of applied toxicology : JAT. 2011;31:515-23.

[23] de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, et al. The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol. 2009;29:5238-50.

[24] Beraldo FH, Soares IN, Goncalves DF, Fan J, Thomas AA, Santos TG, et al. Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and

regulates cellular response to ischemia via the prion protein. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013.

[25] Guzman MS, De Jaeger X, Drangova M, Prado MA, Gros R, Prado VF. Mice with selective elimination of striatal acetylcholine release are lean, show altered energy homeostasis and changed sleep/wake cycle. Journal of neurochemistry. 2013;124:658-69.

[26] Assini JM, Mulvihill EE, Sutherland BG, Telford DE, Sawyez CG, Felder SL, et al. Naringenin prevents cholesterol-induced systemic inflammation, metabolic dysregulation, and atherosclerosis in Ldlr(-)/(-) mice. Journal of lipid research. 2013;54:711-24.

[27] Aguiar CJ, Andrade VL, Gomes ER, Alves MN, Ladeira MS, Pinheiro AC, et al. Succinate modulates Ca(2+) transient and cardiomyocyte viability through PKA-dependent pathway. Cell calcium. 2010;47:37-46.

[28] Prado MA, Reis RA, Prado VF, de Mello MC, Gomez MV, de Mello FG. Regulation of acetylcholine synthesis and storage. Neurochemistry international. 2002;41:291-9.

[29] Prado VF, Roy A, Kolisnyk B, Gros R, Prado MA. Regulation of cholinergic activity by the vesicular acetylcholine transporter. The Biochemical journal. 2013;450:265-74.

[30] Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. The Journal of clinical investigation. 1997;100:169-79.

[31] Lyons GE, Schiaffino S, Sassoon D, Barton P, Buckingham M. Developmental regulation of myosin gene expression in mouse cardiac muscle. The Journal of cell biology. 1990;111:2427-36.

[32] Stacy RC, Demas J, Burgess RW, Sanes JR, Wong RO. Disruption and recovery of patterned retinal activity in the absence of acetylcholine. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2005;25:9347-57.

[33] Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. Annual review of physiology. 2003;65:45-79.

[34] Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, et al. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation. 2007;116:258-67.

[35] Amin JK, Xiao L, Pimental DR, Pagano PJ, Singh K, Sawyer DB, et al. Reactive oxygen species mediate alpha-adrenergic receptor-stimulated hypertrophy in adult rat ventricular myocytes. Journal of molecular and cellular cardiology. 2001;33:131-9.

[36] Waypa GB, Marks JD, Mack MM, Boriboun C, Mungai PT, Schumacker PT. Mitochondrial reactive oxygen species trigger calcium increases during hypoxia in pulmonary arterial myocytes. Circulation research. 2002;91:719-26.

[37] Martini JS, Raake P, Vinge LE, DeGeorge BR, Jr., Chuprun JK, Harris DM, et al. Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:12457-62.

[38] Choi DJ, Koch WJ, Hunter JJ, Rockman HA. Mechanism of beta-adrenergic receptor desensitization in cardiac hypertrophy is increased beta-adrenergic receptor kinase. The Journal of biological chemistry. 1997;272:17223-9.

[39] Parsons SM. Transport mechanisms in acetylcholine and monoamine storage. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2000;14:2423-34.

[40] Canaani J, Shenhar-Tsarfaty S, Weiskopf N, Yakobi R, Assayag EB, Berliner S, et al. Serum AChE Activities Predict Exercise Heart Rate Parameters of Asymptomatic Individuals. Neuroscience and Medicine. 2010;1:43-9.

[41] Mann DL, Kent RL, Parsons B, Cooper Gt. Adrenergic effects on the biology of the adult mammalian cardiocyte. Circulation. 1992;85:790-804.

[42] Sailaja BS, Cohen-Carmon D, Zimmerman G, Soreq H, Meshorer E. Stress-induced epigenetic transcriptional memory of acetylcholinesterase by HDAC4. Proceedings of the National Academy of Sciences of the United States of America. 2012;109:E3687-95.

[43] Gold JI, Gao E, Shang X, Premont RT, Koch WJ. Determining the absolute requirement of G protein-coupled receptor kinase 5 for pathological cardiac hypertrophy: short communication. Circulation research. 2012;111:1048-53.

[44] Ucar A, Gupta SK, Fiedler J, Erikci E, Kardasinski M, Batkai S, et al. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. Nature communications. 2012;3:1078.

[45] Ferlito M, Fulton WB, Zauher MA, Marban E, Steenbergen C, Lowenstein CJ. VAMP-1, VAMP-2, and syntaxin-4 regulate ANP release from cardiac myocytes. Journal of molecular and cellular cardiology. 2010;49:791-800.

[46] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000;405:458-62.

[47] Shaked I, Meerson A, Wolf Y, Avni R, Greenberg D, Gilboa-Geffen A, et al. MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity. 2009;31:965-73.

Chapter 5

CARDIAC ACETYLCHOLINE REGULATES THE PROGRESSION OF VENTRICULAR REMODELING AND DYSFUNCTION UNDER PATHOLOGICAL CONDITIONS

5 Chapter 5

5.1 Chapter Summary

Autonomic dysfunction is a characteristic of cardiac disease and decreased cholinergic signaling is observed in the early stages of heart failure. We have previously shown that rodent cardiomyocytes produce *de novo* ACh, which is critical in maintaining cardiac homeostasis under physiological conditions. Here, we provide novel data indicating that this non-neuronal cardiomyocyte system is also found in human ventricular cardiomyocytes, which express ChAT and VAChT. Furthermore, VAChT expression is significantly increased in ventricular tissue from heart failure patients, suggesting an increase in cardiomyocyte-specific ACh secretion in disease. Mice with a genetic deletion of cardiomyocyte-specific VAChT displayed enhanced ventricular dysfunction and pathological remodeling in a pharmacological model of heart dysfunction, supporting the importance of this intrinsic cholinergic system in the progression of cardiac remodeling. Mice overexpressing VAChT did not display adverse phenotypes suggesting that the increased VAChT expression observed in human HF may serve a protective role rather than contribute to myocardial damage. The data presented here provide novel information regarding the presence of a NNCS in human cardiomyocytes. It appears to be increased in failing hearts and may play a compensatory role, given the known decrease in parasympathetic activity in heart failure.

5.2 Introduction

It is well established that chronic autonomic sympathetic/parasympathetic imbalance plays a crucial role in the development of heart failure (HF) [1-4]. There appears to be a significant increase in adrenergic signaling in heart failure, even in the initial stages of cardiac remodeling prior to the onset of heart dysfunction [5]. The hyperadrenergic state contributes to cardiac remodeling [5, 6] and this correlates with higher morbidity and mortality [7], likely due to the fact that long-term activation of this process may result in ventricular remodeling [3, 8].

The changes in autonomic control in heart failure also involve decreased parasympathetic tone and are observed early [9-11]. Altered heart rate regulation due to changes in vagal nerve activity has been observed shortly after induction of left ventricular dysfunction, suggesting that alterations in cholinergic signaling in the heart may contribute to HF [12].

Previous work in mice has indicated that genetic disturbance of acetylcholine (ACh) release, by targeting the vesicular acetylcholine transporter (VAChT), causes ventricular dysfunction [13]. In agreement with these data, similar results are also observed in mice with decreased levels of the high-affinity choline transporter [14].

It is surprising that cholinergic activity has such widespread consequences to ventricular function, given the sparse levels of cholinergic innervation outside the atria [15]. In the ventricles, cardiac innervation is mainly restricted to the cardiac conduction system [15, 16]. Recent experiments have provided evidence for one mechanism through which ACh may regulate heart activity involves autocrine/paracrine secretion of non-neuronal ACh by cardiac myocytes [17]. Indeed, ventricular cardiomyocytes possess the machinery for *de novo* production, storage and secretion of ACh [18, 19]. The protein responsible for packaging ACh into exocytic vesicles, the VAChT, is an important component of the cholinergic machinery and is present in vesicles in cardiomyocytes [17], suggesting quantal ACh release from myocytes.

Cardiomyocyte overexpression of the enzyme responsible for the production of ACh, choline acetyltransferase (ChAT), in transgenic mice suggests that increased levels of cardiomyocyte ACh protects against myocardial infarction [20]. Moreover, our group has recently reported that the cardiac non-neuronal cholinergic system (NNCS) plays a critical role in regulating cardiac homeostasis *in vivo* [21]. Our data indicate that inhibition of ACh secretion from cardiomyocytes delays heart rate recovery following exercise and leads to significant ventricular remodeling characterized by molecular alterations [21]. As such, these data suggested that cardiomyocyte-secreted ACh plays a critical role in regulating cardiac function under physiological conditions.

Previous experiments have suggested that transdifferentiation of sympathetic to cholinergic neurons takes place during progression of heart failure, a process that plays a protective role [22]. This compensatory regulation may help to decrease the change observed in parasympathetic activity in heart disease. Although neuronal and non-neuronal ACh may influence diseased states, it is unclear whether the non-neuronal cholinergic machinery is present in human cardiomyocytes and whether it is of any consequence for heart disease. We found that both healthy and diseased human cardiomyocytes express markers of the cholinergic system. Furthermore, left ventricular tissue from human patients with non-ischemic dilated cardiomyopathy (NICM) displayed a significant increase in the expression of VAChT, including VAChT present in cardiomyocytes, when compared to age-matched controls. Changes in VAChT levels have significant impact on ACh secretion [23-26]. Therefore, to determine the consequences of changes in ACh synthesis and secretion, we used genetically-modified mice in which either ChAT or VAChT was selectively deleted in cardiomyocytes. The data obtained with mouse models in this study suggest that compensatory changes in VAChT levels in humans may play a role in mitigating the extent of cardiac remodeling in heart failure.

5.3 Materials and Methods

5.3.1 Animal models

Animals were maintained and cared for according to approved animal protocols at the University of Western Ontario (2008-127) and following Canadian Council on Animal Care guidelines. Only male mice were used for all *in vivo* experiments. Cardiomyocyte-specific VAChT KO or ChAT knockout mice ($VAChT^{Myh6\text{-}Cre\text{-}flox/flox}$; cVAChT; $ChAT^{Myh6\text{-}Cre\text{-}flox/flox}$; cChAT) mice were generated as previously described in a previous study [27]. *ChAT-ChR2-EYFP* mice overexpressing the VAChT [25, 28] (B6.Cg-Tg(Chat-COP4*H134R/EYFP,Slc18a3)6Gfng/J) and have previously been characterized [25, 29].

5.3.2 Human tissue samples

Left ventricular tissue samples were obtained from the Duke Human Heart Repository (Durham, NC). LV myocardium was obtained from seven heart failure patients with non-ischemic dilated cardiomyopathy (NICM) and age-matched non-failing individuals. LV tissue was flash frozen for RNA and protein extraction and OCT embedded for immunostaining. The use of these samples was approved by the Institutional Review Board at Duke University Medical Center (Durham, NC).

5.3.3 Angiotensin II infusion

Angiotensin II (Sigma-Aldrich; A9525) was dissolved in sterile saline at a dose of 3 mg/kg/day in osmotic pumps (Model 1002, Alzet). The pumps were implanted subcutaneously and Ang II was infused for 2 weeks following which the mice were housed for an additional two weeks to allow remodeling prior to experimental use.

5.3.4 Echocardiography

M-mode echocardiography was performed in both $VAChT^{flox/flox}$ and $VAChT^{Myh6\text{-}Cre\text{-}flox/flox}$ mice following Ang II treatment. Left ventricular (LV) and right ventricular (RV) ejection fraction and fractional shortening were measured using the Vevo 2100 ultrasound imaging system (Visual Sonics, Canada). Briefly, 2-dimensional images of the heart were obtained in short-axis view using a dynamically focused 40 MHz probe. The M-mode cursor was positioned perpendicular to the LV anterior and posterior walls. The LV internal end-diastolic dimension (LVIDd) and LV internal systolic dimension (LVIDs) were measured from M-mode recordings. LV ejection fraction was calculated as: EF (%) = $[(LVIDd)^3 - (LVIDs)^3]/(LVIDd)^3 \times 100$. Fractional shortening was calculated as: FS (%) = (LVIDd-LVIDs)/LVIDd×100. The M-mode measurements of the left ventricular ejection fraction and fractional shortening were averaged from 3 cycles.

5.3.5 ROS measurement

ROS levels were measured using the MitoSOX Red superoxide indicator (Invitrogen) as previously described [30].

5.3.6 Protein oxidation levels

Hearts from saline-treated or Ang II-treated mice were isolated, fixed and paraffin-embedded. 5 μm tissue sections were obtained and oxidized protein levels were analyzed using the OxyIHC Oxidative Stress Kit (Millipore) following the manufacturer's directions.

5.3.7 Histological analysis

5 μm sections were obtained from saline-treated and Ang II-treated $VAChT^{flox/flox}$ and $VAChT^{Myh6\text{-}Cre\text{-}flox/flox}$ mice. The tissue sections were stained with hematoxylin and eosin (H&E) using standard procedures. Light microscopic images were acquired at 20x magnification at distinct locations within the left ventricular free wall.

Cardiomyocyte cell surface area was measured using H&E stained sections where the cardiomyocyte edges were distinctly observable. Cell surface was measured in sections obtained from at least 4 mice/genotype.

5.3.8 Cardiac fibrosis

5 μm sections obtained from VAChT$^{flox/flox}$ and VAChT$^{Myh6\text{-}Cre\text{-}flox/flox}$ mice following Ang II treatment were stained with Trichrome C using standard procedures to analyze extent of cardiac fibrosis. Light microscopic images were acquired at 20x magnification at separate locations within the left ventricular wall to analyze both interstitial and perivascular collagen deposition was analyzed.

5.3.9 qPCR/RT-PCR

RNA was extracted from human left ventricular tissue or whole hearts and cDNA was synthesized as previously described [30]. qPCR for VAChT, β-MHC, and ANP was performed as previously described [30]. Actin and GAPDH were used to normalize qPCR results for mice and humans, respectively. Primer sequences are available upon request.

5.3.10 Immunoblotting

Protein was extracted from human left ventricular tissue using ice-cold modified RIPA buffer. 50 μg of protein was separated using SDS-PAGE and transferred to PVDF membranes, which were probed with anti-VAChT antibody (1:500; Synaptic Systems). α-actinin (1:2000; Sigma-Aldrich) was used as a loading control.

5.3.11 Immunostaining

Adult cardiomyocytes were subjected to an immunostaining protocol as previously described [31]. Cells were co-stained with anti-ChAT (1:100; Abcam) and anti-α-actinin (1:200; Sigma-Aldrich) antibodies. α-actinin labeled cells were used to measure cardiomyocyte cell surface area. Images were acquired using the Zeiss LSM 510 Meta confocal system.

For human LV tissue, 5 μm sections were subjected to the immunofluorescence protocol as previously described [31]. The tissue was incubated with anti-VAChT (1:50; Synaptic Systems) and anti-α-actinin (1:500; Sigma-Aldrich). Images were acquired using the 63x objective on the Zeiss LSM 510 Meta confocal system.

5.3.12 Measurement of ACh secretion

Acetylcholine release was measured using the Choline/ACh Quantification Kit (Biovision) as previously described [27, 32]. Briefly, atrial tissue was isolated from wild-type and *ChAT-ChR2-EYFP* mice and incubated in Tyrode's solution containing 100 μM pyridostigmine bromide (Sigma-Aldrich; P9797) at 37ºC for 2 hours. The solution was collected and centrifuged at 10,000 RPM for 5 minutes at 4ºC and the resulting supernatant was collected and placed on ice. ACh concentration was determined using the colorimetric Choline/ACh Quantification Kit (Absorbance 570 nm). Each sample was assayed in duplicate and experiments were conducted using at least 3 mice/genotype.

5.3.13 Heart rate and blood pressure measurement

Systolic and diastolic blood pressures as well as heart rate were recorded from conscious animals using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA) as previously described [33].

5.3.14 Electrocardiography

Radiofrequency telemeters were implanted in wild-type and *ChAT-ChR2-EYFP* mice subcutaneously and ECG recordings were obtained as previously described [13]. Heart rate was recorded continuously over 24 hours to obtain baseline recordings. In addition, HR recordings were obtained immediately following IP saline injection or following an acute exercise routine, as previously described [27]. In addition, autonomic regulation of cardiac function was analyzed through evaluation of heart rate responses following autonomic blockade using IP injections of atropine (1 mg/kg) and/or propranolol (1 mg/kg).

5.3.15 Statistical analyses

Results for all experiments are provided as mean ± SEM. Either a Student's t-test or a one-way ANOVA with a Tukey's post-hoc test were used to evaluate statistical differences between experimental groups using Graphpad or SigmaStat. $p<0.05$ was considered statistically significant.

5.4 Results

5.4.1 VAChT levels are increased in failing human myocardium

In order to determine whether the NNCS is present in humans, we used ventricular extracts from control individuals and patients with non-ischemic dilated cardiomyopathy (NICM; Table 5.1) and analyzed cholinergic markers. mRNA levels of acetylcholinesterase (AChE) were not altered in control and heart failure individuals (Fig. 5.1A). mRNA level for ChAT were slightly increased in human HF samples compared to age-matched healthy controls (Fig. 5.1B), although this did not reach significance. Immunoblot and immunofluorescence confirmed that ChAT protein levels were not altered in heart failure (Fig. 5.1C, D). In contrast, VAChT mRNA was significantly increased in HF patients (Fig. 5.1E). Immunoblot analysis indicated that VAChT protein levels were also increased in heart failure patients when compared to controls (Fig. 5.1F). The immunofluorescence data suggest that at least part of the increased levels of VAChT originated in cardiomyocytes (Fig. 5.1G). The VAChT antibody utilized here has previously been validated in VAChT KO cardiomyocytes and is known to specifically recognize VAChT in mammalian cardiomyocytes [27].

5.4.2 cChAT mice display altered heart rate regulation and cardiac remodeling

Previous experiments demonstrated that mice with a cardiomyocyte-specific deletion in VAChT display ventricular remodeling and delayed heart rate recovery following activity [27]. However, VAChT is part of the major facilitator superfamily of transporters, which includes members of the multidrug resistance protein family, and as such can transport other organic substrates, including choline, which can have distinct physiological effects [34, 35]. To further test if the previous phenotypes we described in cVAChT mice are indeed related to lack of ACh release, we utilized cChAT mice and determined whether these mice present a similar phenotype to cVAChT mice.

We isolated adult cardiomyocytes from cChAT mice and found that ChAT transcripts, as determined by RT-PCR, were detected in $ChAT^{flox/flox}$ but not cChAT myocytes (Fig.

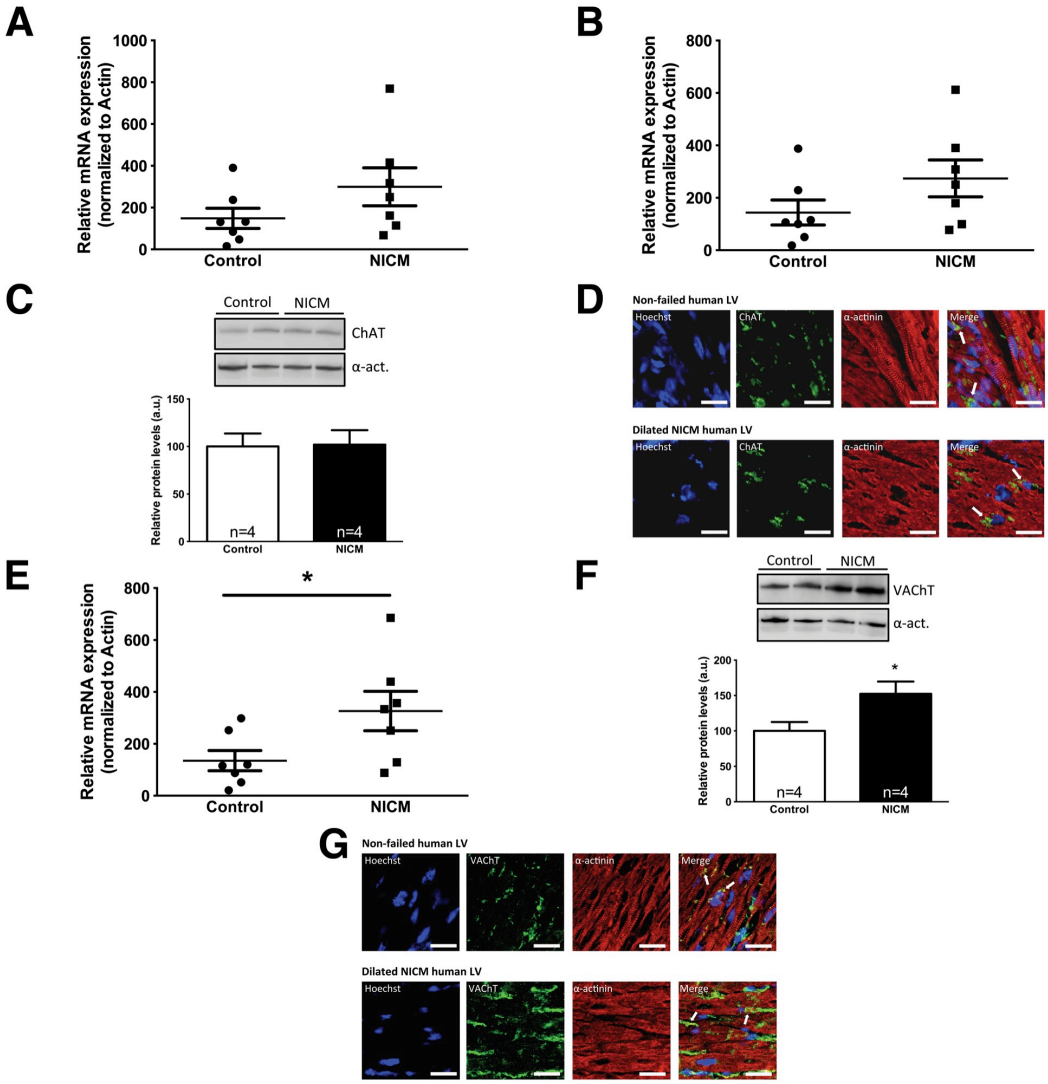
Table 5.1 – Non-ischemic cardiomyopathy (NICM) patient specifics.

ID	Diagnosis	Age
1	End stage dilated NICM; EF 15-20%; s/p AICD; HTN	69
2	Dilated NICM; calcified aortic and mitral valves; IABP	67
3	End stage NICM; hypertrophic dilated CM; inotrope dependent	61
4	Dilated CM; DM2; HTN; HLD	59
5	Dilated NICM; EF <15%; s/p ICD	58
6	End stage dilated NICM; s/p AICD	56
7	End stage NICM; some LVH; possible Dilated CM via MR; RV dysfunction; Lupus	64

EF, ejection fraction; AICD, automatic implantable cardioverter defibrillator; HTN, hypertension; IABP, intra-aortic balloon pump; CM, cardiomyopathy; DM2, diabetes mellitus type 2; HLD, hyperlipidemia; ICD, implantable cardioverter defibrillator; LVH, left ventricular hypertrophy; MR, mitral regurgitation; RV, right ventricle.

Figure 5.1 - Human cardiomyocytes express markers of cholinergic system. (A) mRNA expression for AChE is unaltered in non-ischemic dilated cardiomyopathy (NICM). No difference in ChAT mRNA **(B)** or protein level **(C)** is observed in NICM. **(D)** Perinuclear staining for ChAT is observed in cardiomyocytes from both non-failing and NICM samples. VAChT mRNA **(E)** and protein **(F)** levels are increased in NICM. **(G)** Punctate VAChT staining is observed in the perinuclear region of cardiomyocytes and increased in NICM samples. Data are represented as the mean ± SEM. $n \geq 4$ mice for all experiments. $^{*}p<0.05$.

Figure 5.1 - Human cardiomyocytes express markers of cholinergic system.



5.2A). Staining for ChAT in murine cardiomyocytes showed a pattern that was similar to the one we previously reported, with mainly nuclear and perinuclear staining of ChAT (Fig. 5.2B). This stained was completely abolished in cChAT mice by immunofluorescence analysis (Fig. 5.2B) confirming the elimination of ChAT in isolated ventricular cardiomyocytes.

Previous experiments showed abnormal heart rate regulation in both cVAChT and cChAT mice under restraint conditions [27]. Further experiments in this study with cChAT mice revealed no alterations in heart rate response under baseline conditions over 24 hours in a home-cage environment, as measured via radio-frequency ECG telemetry (Fig. 5.2C). However, these mutant mice did exhibit delayed heart rate recovery following intraperitoneal injection of saline, which was used as a stressor (Fig. 5.2D). Furthermore, as compared to $ChAT^{flox/flox}$ animals, cChAT mice exhibited a greater initial increase in heart rate as well as a delay in heart rate recovery following an acute, low intensity, treadmill exercise test (Fig. 5.2E). These results are essentially the same as we previously reported for cVAChT mice.

In addition, similarly to the previously observed phenotype in cVAChT mice, cChAT animals also displayed an increase in cardiac size (Fig. 5.3A), due to cardiomyocyte hypertrophy (Fig. 5.3B). Furthermore, ChAT knockout myocytes showed a tendency for increased expression of β-MHC ($p=0.07$) and a significant increase in ANP expression (Fig. 5.3C), both markers of cardiac remodeling. In addition, similar to cVAChT myocytes, cChAT myocytes also showed a significant increase in mitochondrial superoxide levels (Fig. 5.3D), indicative of increased oxidative stress and remodeling. Control experiments confirmed that these molecular changes and cardiomyocyte remodeling are absent in Myh6-Cre mice [27]. All the phenotypes uncovered in cChAT mice were also previously observed in cVAChT mice, leading us to conclude that these alterations are due to either lack of ACh synthesis in cChAT mice or lack of ACh release in cVAChT mice [27].

Figure 5.2 - cChAT mice display altered heart rate regulation. (A) ChAT transcript is expressed in $ChAT^{flox/flox}$ myocytes but not in conditional ChAT knockout (cChAT) myocytes. **(B)** Immunofluorescence in adult cardiomyocytes confirms deletion of ChAT at the protein level in cChAT cardiomyocytes. **(C)** Heart rate over 24 hours in awake, free moving mice is unaltered between $ChAT^{flox/flox}$ and cChAT mice in their home cage. **(D)** cChAT mice exhibit a delay in heart rate recovery following gentle restraint and IP saline injection. **(E)** cChAT mice exhibit a greater increase in initial heart rate and delayed recovery to baseline levels following exercise, as compared to $ChAT^{flox/flox}$ controls. $n \geq 7$ for all experiments. Data are represented as mean ± SEM. *$p<0.05$ vs. control mice.

Figure 5.2 - cChAT mice display altered heart rate regulation.

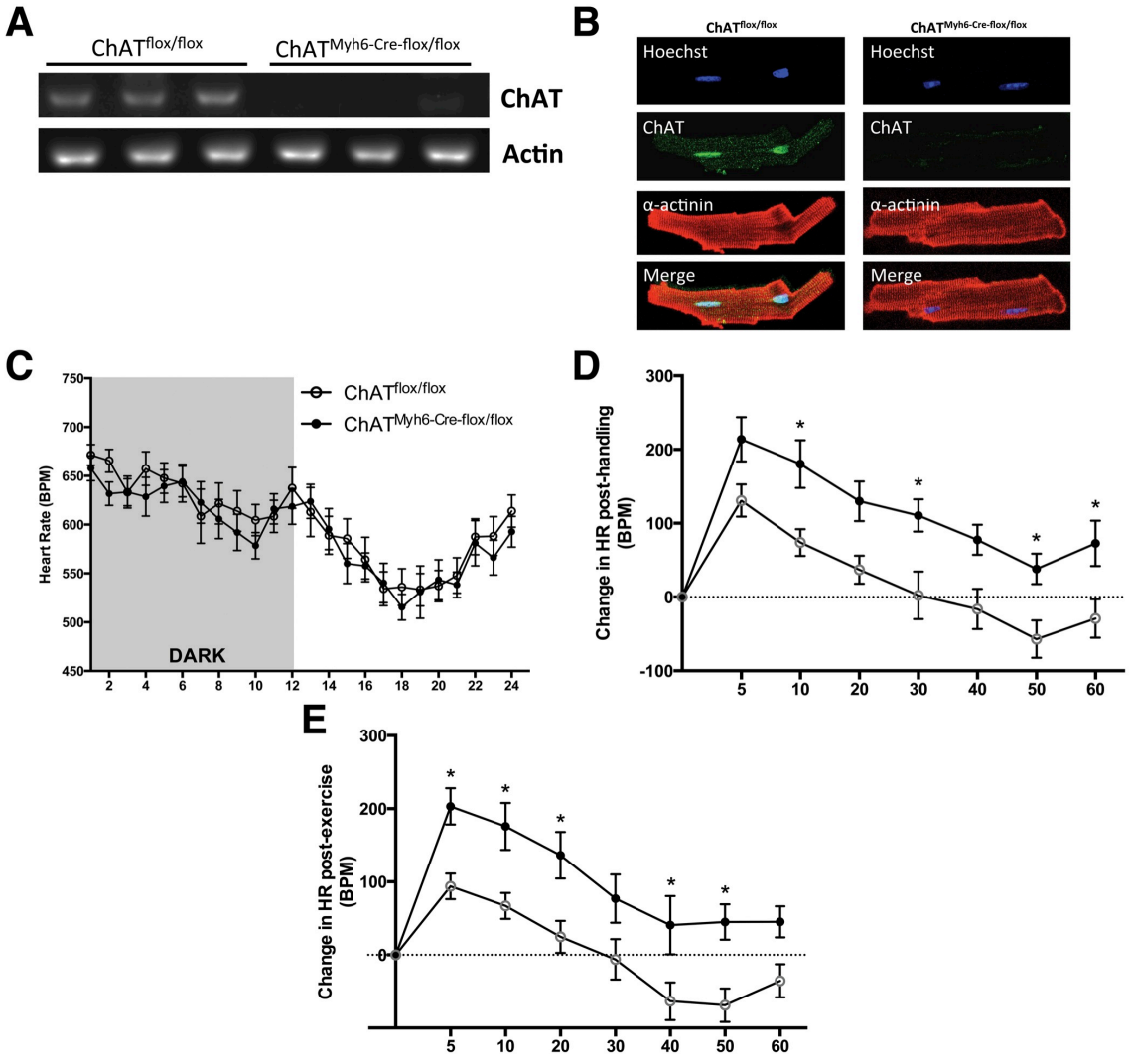
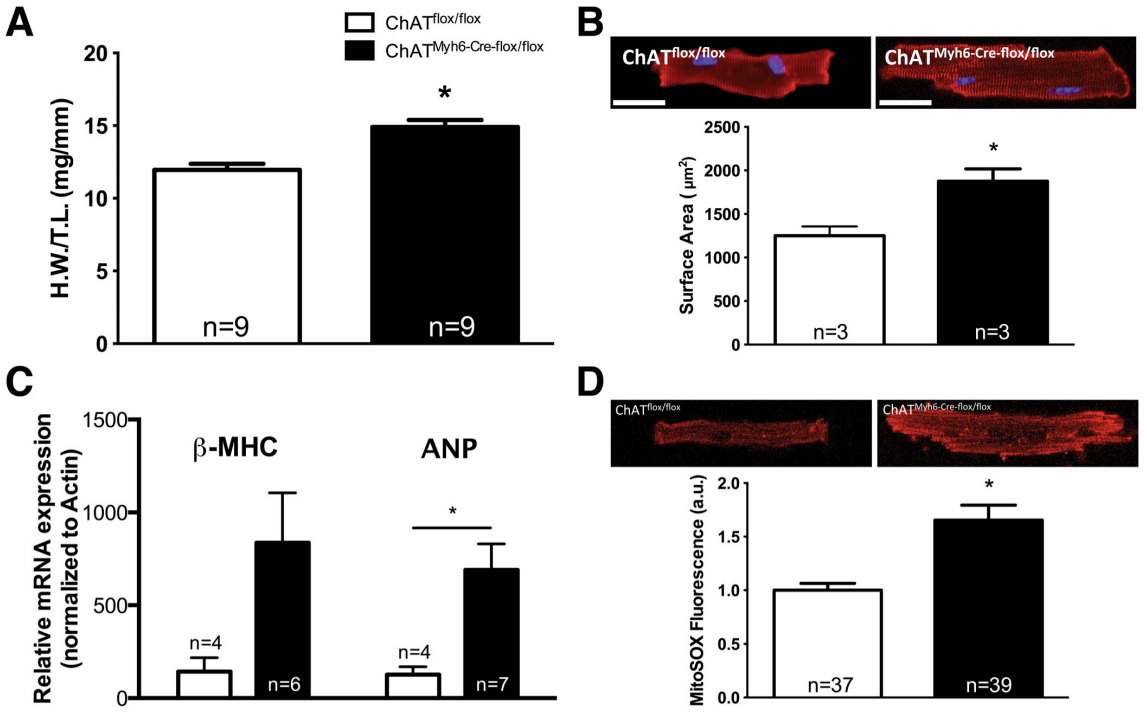


Figure 5.3 - cChAT mice exhibit ventricular remodeling and hypertrophy. (A) cChAT mice display cardiac hypertrophy, as measured through heart weight (normalized to tibia length, n=number of cells). **(B)** Isolated cChAT cardiomyocytes exhibit a significant increase in surface area, as compared to $ChAT^{flox/flox}$ cells (n=number of mice). **(C)** Expression of the cardiac stress marker, β-MHC, shows a tendency for increase in cChAT myocytes while the expression of ANP is significantly increased in cChAT cardiomyocytes, as compared to control cells (n=number of mice). **(D)** ROS levels in cChAT myocytes, as measured using the MitoSOX superoxide indicator, are significantly increased (n=number of cells; at least 3 mice/genotype). Data are represented as the mean ± SEM. *$p<0.05$.

Figure 5.3 - cChAT mice exhibit ventricular remodeling and hypertrophy.



5.4.3 cVAChT mice exhibit increased angiotensin II-mediated cardiac remodeling

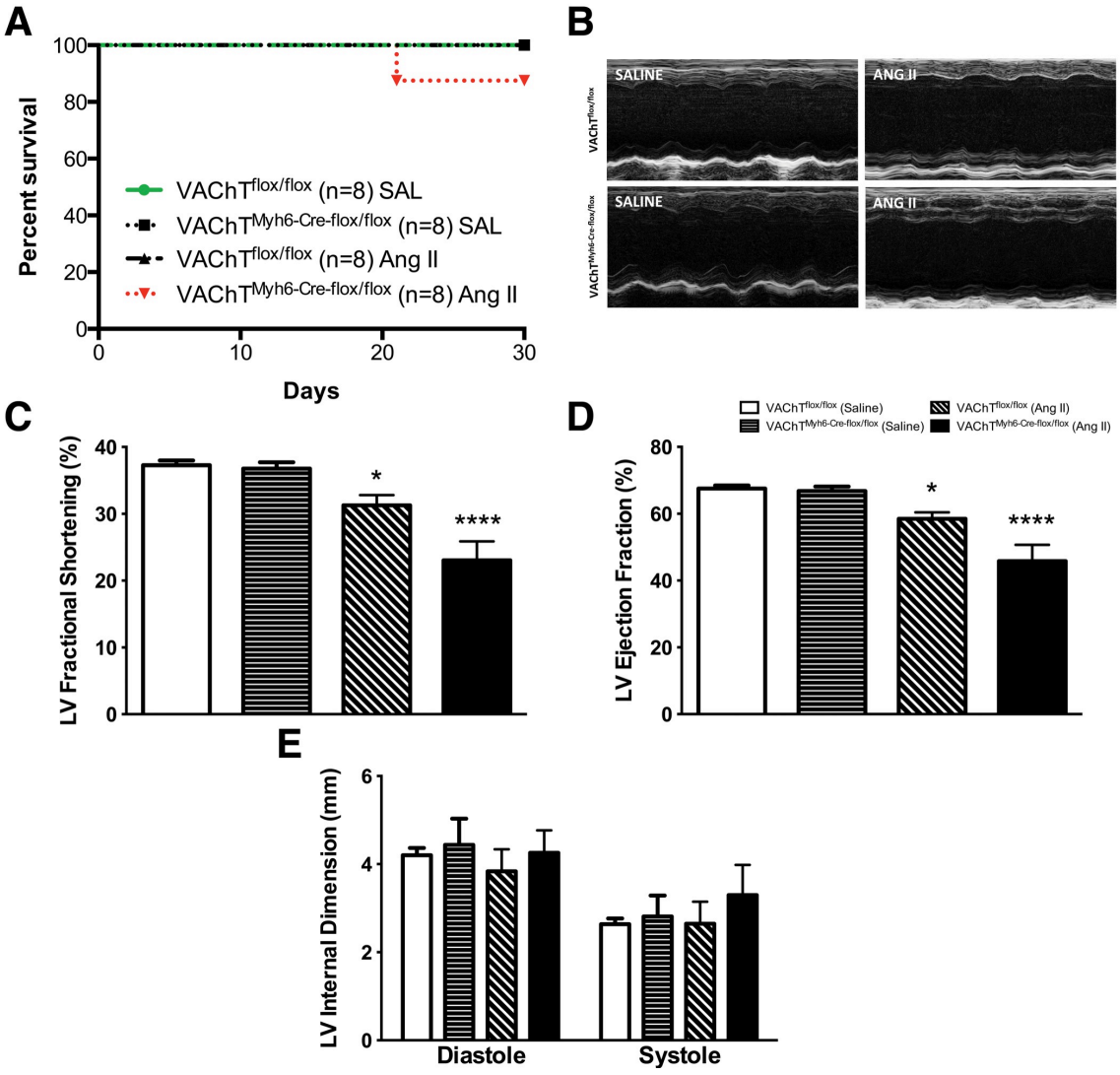
Our data suggest that the cholinergic cardiomyocyte system has physiological impact on heart function in mice. Therefore, our goal was to determine whether cardiomyocyte VAChT contributes to remodeling under stress. To clarify whether changes in the expression levels of VAChT, which can effectively regulate ACh release [23-26], could impact pathological remodeling, we focused our experiments on cVAChT mice chronically treated with angiotensin II (Ang II). Ang II is a potent vasoconstrictor, which activates AT_1 receptors on vascular smooth muscle cells [36, 37] and can thereby induce ventricular remodeling by increasing cardiac afterload. Furthermore, Ang II-mediated signaling has also been shown to directly induce hypertrophy and molecular remodeling in ventricular cardiomyocytes [38-40]. We chose pharmacological treatment instead of surgical induction of heart failure in mice in an attempt to produce limited cardiac dysfunction, in case the lack of VAChT led to worse outcomes.

Ang II treatment did not lead to significantly increased mortality in cVAChT mice, as compared to control animals (Fig. 5.4A). However, non-invasive analysis of left ventricular hemodynamics using M-mode echocardiography confirmed a significant decrease in LV fractional shortening as well as LV ejection fraction in both control and cVAChT mice following Ang II treatment (Fig. 5.4B, C, D). Importantly, cVAChT mice exhibited a significantly greater decrease in both LV fractional shortening and ejection fraction, as compared to Ang II-treated control mice (Fig. 5.4C, D), thus suggesting that cVAChT mice were more sensitive to Ang II-mediated cardiac dysfunction. Notably, neither genotype displayed LV dilation following Ang II treatment, as the LV internal dimensions were similar across genotypes during both diastole and systole (Fig. 5.4E). The lack of ventricular dilation confirms that we are analyzing the initial stages of ventricular remodeling in these animals, prior to the onset of heart failure and severe decline in contractile function.

In order to determine pathological hallmarks in cVAChT mice that may contribute to the worsened LV dysfunction observed following Ang II treatment, heart weight and

Figure 5.4 - cVAChT mice exhibit enhanced ventricular dysfunction following Ang II treatment. (A) No significant difference in survival is observed following Ang II treatment. **(B)** Representative images of M-mode echocardiography suggest a greater decline in cardiac function in cVAChT mice following Ang II treatment, as compared to control mice. The LV fractional shortening **(B)** and ejection fraction **(C)** is significantly decreased in both $VAChT^{flox/flox}$ and cVAChT mice, with the mutant mice displaying a more significant decrease in both parameters. **(D)** The LV internal dimensions during diastole and systole are not altered in either genotype following Ang II treatment. Data are represented as the mean ± SEM. $n \geq 7$ mice for all experiments. *$p<0.05$, ****$p<0.0001$.

Figure 5.4 - cVAChT mice exhibit enhanced ventricular dysfunction following Ang II treatment.



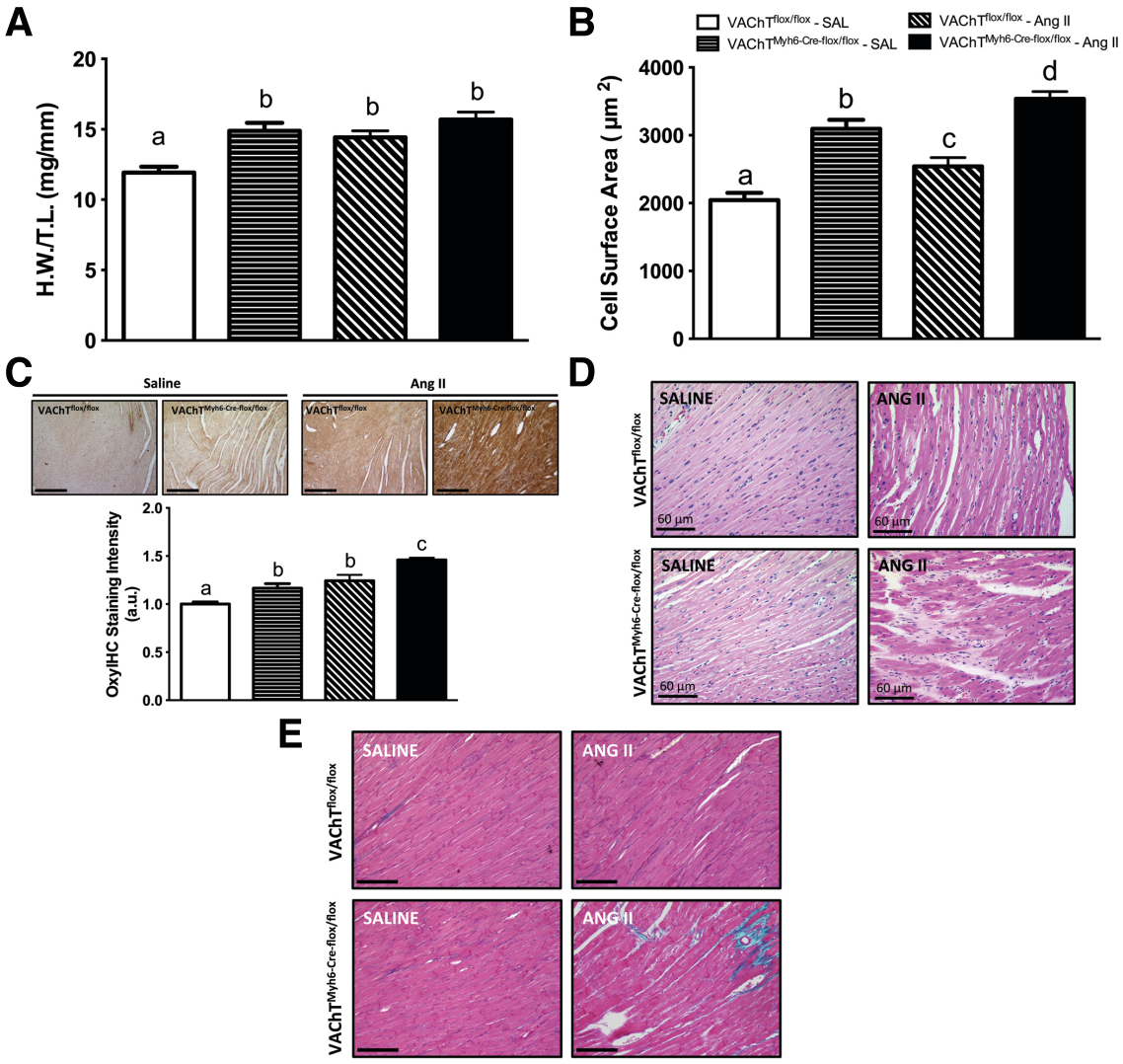
cardiomyocyte surface area were analyzed. VAChT$^{flox/flox}$ control mice displayed an increase in heart weight following Ang II treatment as compared to saline-treated mice (Fig. 5.5A). Conversely, cVAChT mice displayed baseline cardiac hypertrophy with saline treatment alone, as previously described [27]; however, as compared to saline-treated animals, Ang II treatment did not induce a further significant increase in heart weight in cVAChT mice (Fig. 5.5A). Notably, an analysis of cardiomyocyte surface area, *in situ*, revealed that Ang II-treated cVAChT mice exhibit a significantly greater cardiomyocyte hypertrophic response than Ang II-treated control mice (Fig. 5.5B).

Since chronic Ang II exposure can lead to increased reactive oxygen species (ROS) production and cardiomyocyte death [41-43], we analyzed changes in the myocardium. Further analysis revealed genotype-dependent changes in oxidative stress, as measured by the levels of oxidized proteins in the myocardium. Specifically, under baseline conditions with saline treatment, there was an increase in oxidative stress in cVAChT mice, as compared to control mice (Fig. 5.5C), consistent with our previous observations [27]. Furthermore, Ang II treatment led to a greater increase in oxidative stress in the myocardium in cVAChT mice as compared to Ang II-treated control mice (Fig. 5.5C).

In addition to the changes in oxidative stress, Ang II-treated cVAChT mice displayed a greater disruption of myocardial structure, as compared to VAChT$^{flox/flox}$ control mice (Fig. 5.5D). In order to determine the potential for ECM remodeling, Trichrome C staining was performed to analyze collagen deposition. In VAChT$^{flox/flox}$ mice, no fibrosis was observed following treatment with Ang II; however, in cVAChT mice, prominent fibrosis due to increased interstitial and perivascular collagen deposition was observed (Fig. 5.5E). Together, these results suggest that decreased levels of VAChT in cardiomyocytes, without alterations in the parasympathetic nerves [27], can negatively influence cardiac remodeling in response to a pathological insult.

Figure 5.5 - cVAChT mice display increased ventricular hypertrophy and remodeling. (A) Ang II treatment led to a significant increase in HW/TL ratio in $VAChT^{flox/flox}$ control mice while cVAChT mice did not display cardiac hypertrophy. **(B)** Ang II treatment led to a significant increase in cardiomyocyte hypertrophy in both genotypes; however, an even greater hypertrophic response was observed in cVAChT mice. **(C)** Oxidative protein levels were increased following Ang II treatment. cVAChT mice displayed higher levels of protein oxidation than the control animals, as evidenced by quantification of staining intensity. **(D)** $VAChT^{flox/flox}$ control mice exhibited myocardial disruption following Ang II treatment, a response that was further enhanced in cVAChT mice. **(E)** Trichrome C staining revealed no increase in fibrotic response in control animals following Ang II treatment; however, cVAChT mice exhibited both interstitial and perivascular fibrosis. Data are represented as the mean ± SEM. $n \geq 4$ mice for all experiments. Letters indicate significant differences among groups ($p<0.05$).

Figure 5.5 - cVAChT mice display increased ventricular hypertrophy and remodeling.

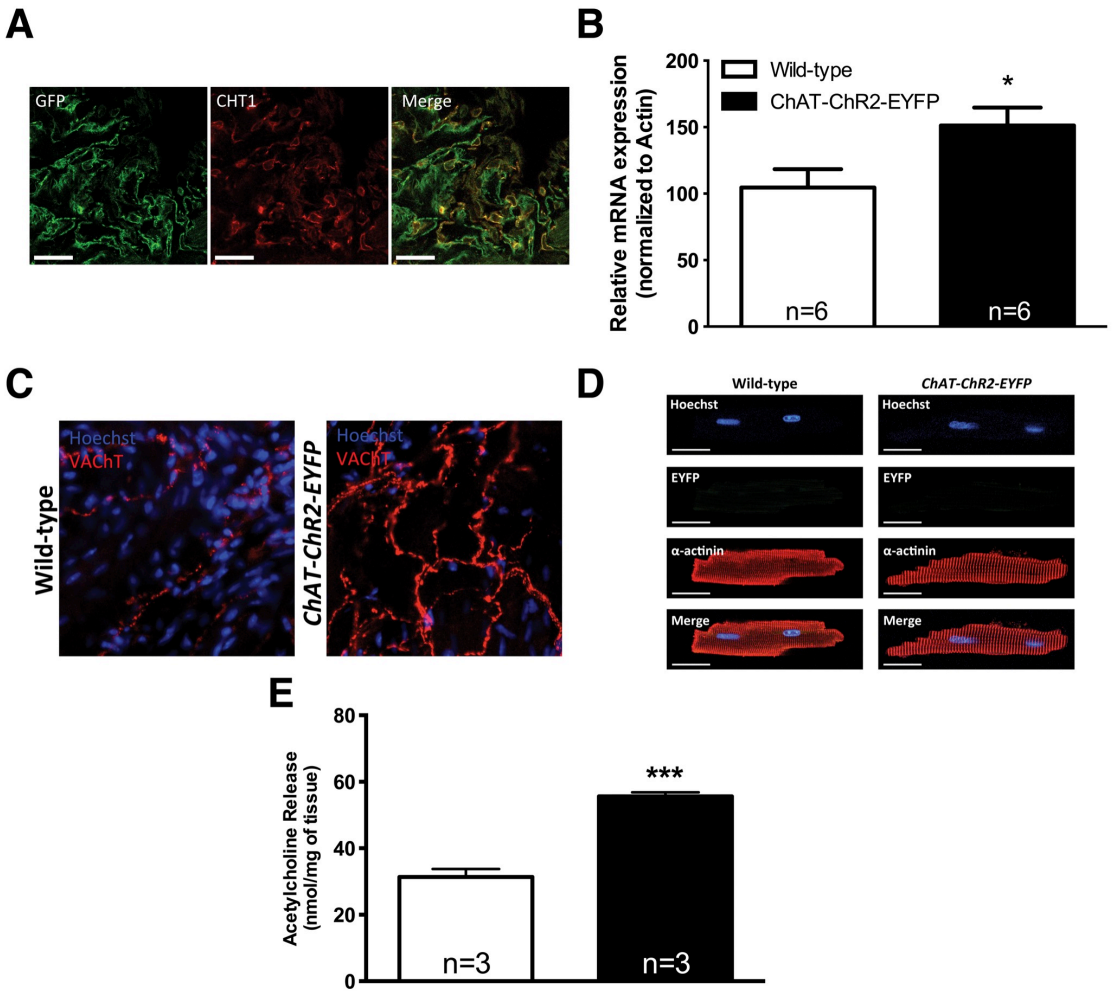


5.4.4 Increased VAChT expression does not disturb cardiac function

Although decreased levels of VAChT in either nerve terminals [13, 30] or cardiomyocytes [27] seem to influence cardiac remodeling, whether increased VAChT levels may affect cardiac function is unclear. Therefore, we used a newly described mouse line overexpressing VAChT using BAC transgenics (*ChAT-ChR2-EYFP)* for these experiments [44]. This particular line was generated to express channelrhodopsin-2 (ChR2) in cholinergic neurons using a ChAT BAC; however, because the VAChT gene is present in the first intron of the ChAT gene, the transgene leads to VAChT overexpression [25]. Previous experiments showed an approximately 25-fold increase in VAChT mRNA levels in the brain; however, there was only a 3-5 fold increase in protein levels, depending on the brain region in this mouse line [25]. Because expression of the gene is regulated by the endogenous promoter regions in the BAC, we first confirmed whether the atria of *ChAT-ChR2-EYFP* mice overexpressed VAChT. Due to the fact that the ChR2 protein is tagged with EYFP, we performed fluorescent immunohistochemistry to confirm the expression of the BAC transgene in atrial tissue. Immunostaining confirmed the expression of EYFP in cholinergic neurons, which were specifically labeled with the cholinergic marker CHT1 (Fig. 5.6A). qPCR analysis revealed that expression of the transgene in the heart led to a modest, but significant, increase in VAChT expression (Fig. 5.6B). Furthermore, immunostaining of atrial tissue confirmed an increase in staining for VAChT thus suggesting an increase in VAChT protein levels in *ChAT-ChR2-EYFP* mice (Fig. 5.6C). Notably, EYFP was not present in ventricular cardiomyocytes, thus suggesting that the BAC transgene is not expressed in these cardiomyocytes (Fig. 5.6D). It is possible that BAC transgene expression may be regulated differently in neuronal vs. non-neuronal tissues. Since the BAC was not expressed in ventricular cardiomyocytes, VAChT would not be upregulated in these cells. Therefore, we measured ACh release from atrial tissue, which contains cholinergic nerve terminals and cell bodies, using a colorimetric assay to quantify ACh secretion [27] and confirmed that the increase in VAChT expression led to a significant increase in ACh release (Fig. 5.6D). The increase in ACh release follows the modest increase in VAChT

Figure 5.6 - Characterization of VAChT expression and ACh release in *ChAT-ChR2-EYFP* hearts. (A) The BAC transgene containing *ChR2-EYFP* is expressed in CHT1-labelled cholinergic neurons in the atria of *ChAT-ChR2-EYFP* mice. **(B)** VAChT expression is significantly increased in *ChAT-ChR2-EYFP* hearts, **(C)** *ChAT-ChR2-EYFP* atria exhibit greater staining for VAChT, as compared to the wild-type mice. **(D)** Staining for *ChR2-EYFP* is absent in isolated, ventricular cardiomyocytes suggesting a lack of BAC expression in these cells (scale bar=25 μm). **(E)** ACh release is significantly increased in *ChAT-ChR2-EYFP* atria. Data are represented as the mean ± SEM. *$p<0.05$ vs. control, ***$p<0.001$ vs. control. n=at least 3 mice/genotype for all experiments.

Figure 5.6 - Characterization of VAChT expression and ACh release in *ChAT-ChR2-EYFP* hearts.



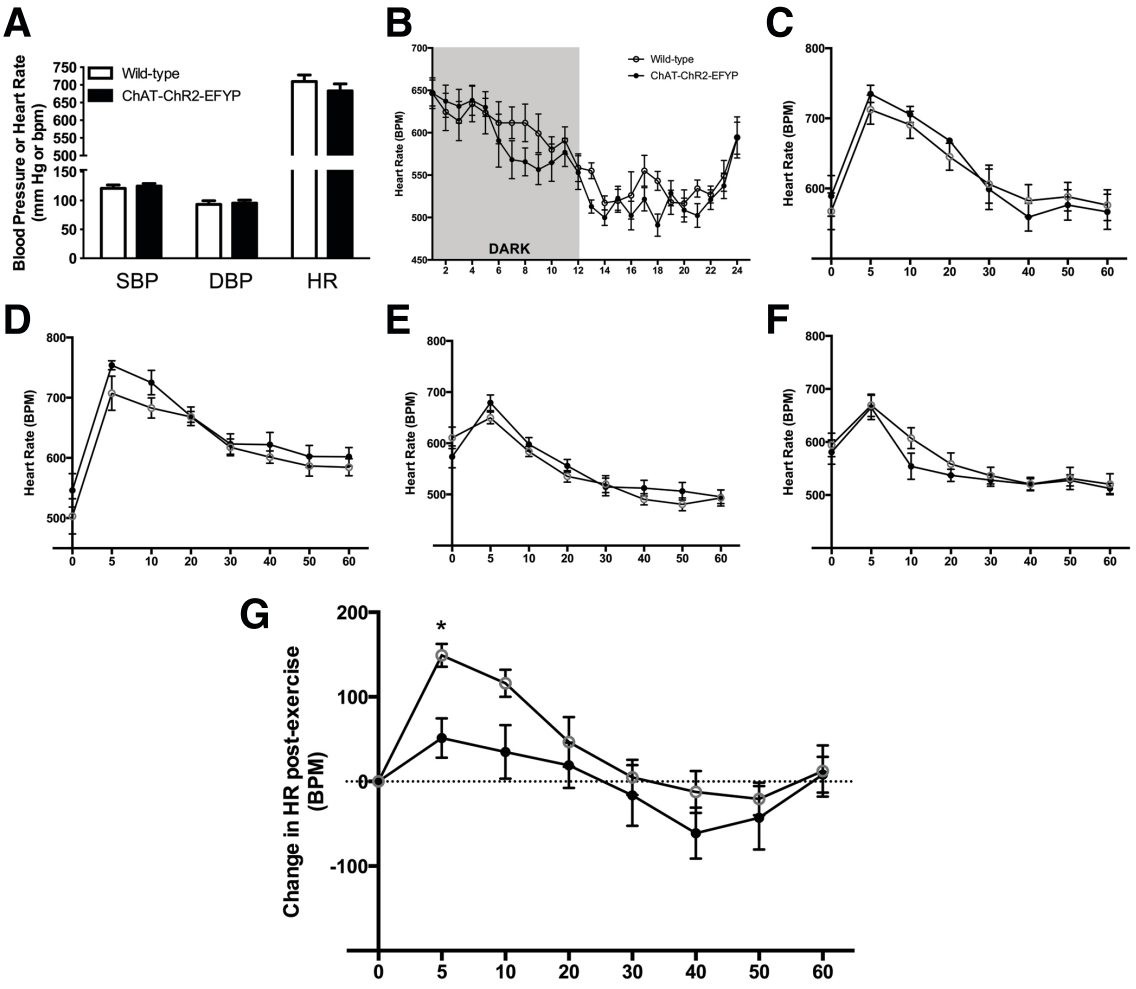
levels. Therefore, it seems that the ChAT BAC used here is not as effective in increasing the expression of VAChT in parasympathetic neurons as it is in the brain.

In order to determine whether this modest increase in systemic cholinergic tone leads to physiological changes in these mice, we performed tail-cuff analysis to measure blood pressure and heart rate. No difference in either heart rate or blood pressure was observed between the two genotypes, as measured using the CODA tail-cuff system (Fig. 5.7A). Electrocardiographic analysis was also performed using radio frequency telemeters to analyze heart rate responses under both baseline conditions as well as following administration of pharmacological agents to determine whether there was autonomic dysregulation in these mice. The baseline heart rate in *ChAT-ChR2-EYFP* mice, as measured over 24 hours, did not differ from the control counterparts (Fig. 5.7B). Furthermore, the response to an IP injection of saline was not altered in transgenic mice (Fig. 5.7C), suggesting that the stress-related response to handling was not affected by the modest increase in cholinergic tone. In addition, in order to measure autonomic imbalance in these mice, we gave a bolus dose of various pharmacological drugs and monitored the HR response in these animals. There was no difference in heart rate response between the control and *ChAT-ChR2-EYFP* mice following administration of a bolus dose of the muscarinic receptor antagonist, atropine (Fig. 5.7D), the β-adrenergic receptor antagonist, propranolol (Fig. 5.7E), or atropine and propranolol together (Fig. 5.7F). These data suggest that there is no major difference in autonomic regulation of heart rate. Furthermore, the intrinsic heart rate in *ChAT-ChR2-EYFP* mice is also unaltered, as evidenced by the heart rate response following inhibition of all autonomic regulation. In contrast, when the *ChAT-ChR2-EYFP* mice were subjected to an acute, low-intensity treadmill test, the initial increase in heart rate following the exercise was significantly reduced as compared to control counterparts (Fig. 5.7G), suggesting that under increased physiological stress overexpression of VAChT may play a role.

To determine whether this increase in cholinergic tone leads to changes in left ventricular hemodynamics, we used the Millar catheter technique to assess cardiac function in live animals. Analysis of LV parameters in anesthetized animals was done

Figure 5.7 - Heart rate response to exercise is attenuated in *ChAT-ChR2-EYFP* mice. **(A)** No difference in blood pressure or heart rate is observed between wild-type and *ChAT-ChR2-EYFP* mice. **(B)** Heart rate over 24 hours in awake, free moving mice is unaltered between WT and *ChAT-ChR2-EYFP* mice in their home cage. **(C)** Heart rate recovery following gentle restraint and IP saline injection is similar in WT and *ChAT-ChR2-EYFP* mice. The heart rate response of both wild-type and *ChAT-ChR2-EYFP* mice is similar following a bolus dose of atropine **(D)**, propranolol **(E)** or atropine and propranolol together **(F)**. **(G)** The initial increase in heart rate following exercise is attenuated in *ChAT-ChR2-EYFP* mice as compared to wild-type controls. $n \geq 7$ mice for all experiments. Data are represented as the mean ± SEM. $^{*}p<0.05$ vs. control mice.

Figure 5.7 - Heart rate response to exercise is attenuated in *ChAT-ChR2-EYFP* mice.



under both baseline conditions and following the administration of isoproterenol (0.5 μg i.p.). All of the hemodynamic parameters measured using the Millar technique were similar between the wild-type and *ChAT-ChR2-EYFP* mice, both under baseline conditions as well as following a bolus dose of isoproterenol (Table 5.2). These results suggest that, under baseline conditions, at least without the presence of an external stressor, modest increased cholinergic tone due to increased VAChT expression does not alter LV function.

Table 5.2 – Hemodynamic parameters for wild-type (n=7) and *ChAT-ChR2-EYFP* (n=7) mice under baseline and following isoproterenol stimulation.

Parameter	Baseline		Isoproterenol	
	Wild-type	***ChAT-ChR2-EYFP***	***Wild-type***	***ChAT-ChR2-EYFP***
HR (bpm)	324 ± 28	260 ± 16	636 ± 33	588 ± 30
LVSP (mm Hg)	112.1 ± 3.3	107.3 ± 4.1	105.8 ± 9.4	105.4 ± 5.1
LVEDP (mm Hg)	9.3 ± 1.7	8.0 ± 1.4	1.3 ± 1.3	0.7 ± 1.0
$+dP/dT_{max}$ (mmHg/s)	9190 ± 509	8078 ± 383	14940 ± 1116	16802 ± 726
$-dP/dT_{min}$ (mmHg/s)	-8516 ± 608	-8246 ± 491	-10623 ± 1068	-10231 ± 795
Contractility index (s^{-1})	178.3 ± 8.3	164.6 ± 1.5	329.9 ± 20.5	339.6 ± 12.3

HR, heart rate; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; $+dP/dT_{max}$, maximum first derivative of the change in left ventricle pressure; $-dP/dT_{min}$, minimum first derivative of the change in left ventricle pressure.
Values are represented as mean ± SEM.

5.5 Discussion

Substantial data suggest that increasing cholinergic signaling in both animal models of HF as well as human patients can reduce morbidity and mortality associated with ventricular dysfunction. In an animal model of heart failure, conjunctive treatment with vagal nerve stimulation and β-blockade therapy has been shown to improve cardiac contractility and animal survival [45, 46]. Furthermore, both cardiac remodeling and mortality were reduced in animal models of HF following chronic treatment with the cholinesterase inhibitor, donepezil [47, 48]. Finally, increasing extracellular ACh levels through administration of a peripheral quaternary cholinesterase inhibitor, pyridostigmine, led to greater vagal control of the heart and reduced ventricular dysfunction in rats with HF [49]. Importantly, enhancing cholinergic tone also appears to have positive consequences in human HF patients. A clinical trial investigating vagal stimulation via an implantable system led to an improvement in their NYHA class score as well as a decrease in LV end-systolic volume [50]. Additionally, in a nationwide cohort study in Sweden, the use of cholinesterase inhibitors in Alzheimer's disease patients led to a 34% reduction in risk of myocardial infarction and death [51].

The mechanisms through which treatment with cholinesterase inhibitors can induce protective effects have yet to be explored. However, it is likely that at least some of the positive effects observed in response to increased cholinergic activity are due to enhanced signaling of the cardiac NNCS. The notion that cardiomyocyte-derived ACh serves a protective role in cardiac disease is supported by data presented here which suggest that VAChT is upregulated in cardiomyocytes from failing human myocardium. To the best of our knowledge, this is the first report to not only demonstrate the existence of a non-neuronal cholinergic system in human cardiomyocytes, but also to report an increase in VAChT expression in heart failure. This increase in VAChT levels in human patients with NICM and HF hints at the possibility that ventricular myocytes may be able to increase cardiac ACh secretion. We propose that this increase in VAChT is not deleterious, and may help to offset some of the remodeling in heart failure. Obviously,

this modest increase in VAChT expression does not seem to be enough to prevent remodeling.

We utilized mice lacking the ability to produce ACh (cChAT) and confirmed that cardiomyocyte ACh is essential in maintaining cardiac homeostasis, similar to the previously observed phenotype in cVAChT mice [27]. These experiments strongly suggest that the phenotypes previously observed in cVAChT mice are essentially due to lack of ACh secretion. In order to further dissect the importance of the NNCS in cardiac remodeling, we chronically treated cVAChT mice with Ang II. Chronic treatment with Ang II led to enhanced remodeling characterized by increased oxidative stress and myocyte hypertrophy. However, LV dysfunction was more pronounced in VAChT-deleted mice. This was likely due to increased disruption of myocardial structure and increased fibrotic response compared to control mice, both of which contribute to decreased ventricular compliance. The increased cardiac remodeling and hypertrophy observed in cVAChT mice following induction of cardiac disease suggests that the NNCS plays a critical role in attenuating the ventricular remodeling response.

Importantly, analysis of pathological remodeling was performed at 1 month following Ang II infusion. This time point was selected in order to determine the importance of the NNCS in the early stages of ventricular remodeling, prior to the onset of heart failure. Control animals did not display enhanced ECM remodeling and fibrotic response following Ang II treatment. In fact, the myocardial structure appeared to be similar to that observed in saline-treated animals. These data suggest that, in the absence of cardiomyocyte-derived ACh, disruption of the myocardium is accelerated following activation of initial neurohumoral responses. Importantly, there is a distinct possibility that the increased oxidative stress observed in cVAChT mice leads to cardiomyocyte death, which may serve as a precursor for the accelerated ventricular remodeling and dysfunction associated with chronic Ang II infusion. The molecular mechanisms that regulate this enhanced remodeling response have yet to be elucidated and will be pursued in future studies.

In agreement with the current study, previous work from other groups has also confirmed the importance of the NNCS in cardiac disease. Studies have shown that ChAT KO HL-1 cells, derived from murine atrial cardiac tissue, exhibit decreased viability in response to chemical hypoxia, likely due to decreased levels of cellular ATP in these cells [52]. Furthermore, these cells appear to be more sensitive as acute treatment with norepinephrine leads to greater production of reactive oxygen species (ROS) as well as increased apoptotic response in ChAT KO cells [52]. Conversely, overexpression of the enzyme responsible for the synthesis of ACh, choline acetyltransferase (ChAT-Tg), specifically in cardiomyocytes, attenuates ventricular remodeling and increases survival following myocardial infarction [20]. These data further highlight the importance of the cardiac NNCS in the regulation of cardiac function, especially following induction of stress, including ischemia.

Although the data presented here suggest that myocardial cholinergic signaling might be increased in human HF, it is unclear whether this contributes to the decline in cardiac function or played a protective role. *ChAT-ChR2-EYFP* mice were utilized to determine whether increased cholinergic signaling led to adverse cardiac effects. Due to the BAC driven overexpression of VAChT, these mice do not seem to fully recapitulate the overexpression phenotype we observed in humans. First, it seems that the BAC is not expressed in isolated cardiomyocytes (Fig. 6D); therefore, VAChT may not be upregulated in the ventricles of these animals. Moreover, the level of overexpression in cholinergic parasympathetic nerve terminals is much less than what we previously reported in the brain [25]. For example, we have previously observed a 20-fold increase in VAChT mRNA levels in the brain, whereas we only detected a 50% increase in expression in the heart (Fig. 6B). Nonetheless, ACh release in atrial tissue follows the increase in VAChT levels, providing a way to follow the consequences of moderately increased levels of ACh release. Interestingly, we observed no changes in inotropy, chronotropy or autonomic regulation of heart rate in these animals. These data suggest that, under physiological conditions, enhancing cholinergic signaling does not alter cardiac function. However, in the presence of physiological stress, increased cholinergic tone can attenuate exercise-induced increase in heart rate. This blunted response to

activity suggests that increased ACh signaling in the heart may lead to greater control of the heart under conditions of increased sympathetic drive, as seen in the disease state. Since enhancing cholinergic signaling in models of HF yields positive results, future studies will utilize these *ChAT-ChR2-EYFP* mice to analyze the progression of ventricular remodeling following induction of cardiac disease.

Importantly, our data indicate that cardiomyocyte-secreted ACh plays a critical role in regulating the onset and progression of LV dysfunction in mice. It is possible that, in cardiac disease, the expression of markers of the cholinergic system in cardiomyocytes is upregulated, which would help to restore cholinergic signaling to baseline levels following vagal withdrawal. In addition, cardiomyocyte ACh may counteract the effects of increased sympathetic drive, which is observed in heart disease. The fact that human cardiomyocytes display increased expression of VAChT in HF gives further support to this notion and may provide a mechanism through which enhancing cholinergic tone in animal models and human patients can have its beneficial effects.

Notably, a global increase in VAChT expression, and thus ACh release, does not lead to adverse consequences in mice but rather serves to offset sympathetic signaling under conditions of physiological stress. These results serve as an important precursor to the clinical use of cholinesterase inhibitors in heart failure patients. The safety of cholinesterase inhibitors is well characterized in Alzheimer's disease patients; therefore, modulation of cholinergic signaling may serve as an unexplored clinical avenue for treatment of cardiac disease in humans.

5.6 Acknowledgments

This work was supported by the Heart and Stroke Foundation of Ontario (Grant NA6656 and G-13-0002843), Canadian Institutes for Health Research (Grant MOP-82756 and MOP-89919), Canadian Foundation for Innovation, the Ontario Research Fund and the National Institutes for Health (Grant R03TW008425 from the Fogarty International Center to S.G.). AR is supported by an Ontario Graduate Scholarship. R.G. was supported by a New Investigator Award from the Heart and Stroke Foundation of Canada.

5.7 References

[1] Mann DL. Mechanisms and models in heart failure: A combinatorial approach. Circulation. 1999;100:999-1008.

[2] Braunwald E, Bristow MR. Congestive heart failure: fifty years of progress. Circulation. 2000;102:IV14-23.

[3] Floras JS. Sympathetic nervous system activation in human heart failure: clinical implications of an updated model. J Am Coll Cardiol. 2009;54:375-85.

[4] Massie BM. Is neurohormonal activation deleterious to the long-term outcome of patients with congestive heart failure? I. Introduction. J Am Coll Cardiol. 1988;12:547-50.

[5] Hasking GJ, Esler MD, Jennings GL, Burton D, Johns JA, Korner PI. Norepinephrine spillover to plasma in patients with congestive heart failure: evidence of increased overall and cardiorenal sympathetic nervous activity. Circulation. 1986;73:615-21.

[6] Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature. 2002;415:206-12.

[7] DiPiro JT, Talbert, Robert L., Yee, Gary C., Matzke, Gary R., Wells, Barbara G., Posey, L. Michael. Pharmacotherapy: A Pathophysiological Approach. 7th ed. New York, NY: McGraw-Hill; 2008.

[8] Triposkiadis F, Karayannis G, Giamouzis G, Skoularigis J, Louridas G, Butler J. The sympathetic nervous system in heart failure physiology, pathophysiology, and clinical implications. J Am Coll Cardiol. 2009;54:1747-62.

[9] Binkley PF, Nunziata E, Haas GJ, Nelson SD, Cody RJ. Parasympathetic withdrawal is an integral component of autonomic imbalance in congestive heart failure: demonstration in human subjects and verification in a paced canine model of ventricular failure. J Am Coll Cardiol. 1991;18:464-72.

[10] Grassi G, Seravalle G, Bertinieri G, Turri C, Stella ML, Scopelliti F, et al. Sympathetic and reflex abnormalities in heart failure secondary to ischaemic or idiopathic dilated cardiomyopathy. Clin Sci (Lond). 2001;101:141-6.

[11] Grassi G, Seravalle G, Cattaneo BM, Lanfranchi A, Vailati S, Giannattasio C, et al. Sympathetic activation and loss of reflex sympathetic control in mild congestive heart failure. Circulation. 1995;92:3206-11.

[12] Kinugawa T, Dibner-Dunlap ME. Altered vagal and sympathetic control of heart rate in left ventricular dysfunction and heart failure. The American journal of physiology. 1995;268:R310-16.

[13] Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, et al. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol. 2010;30:1746-56.

[14] English BA, Appalsamy M, Diedrich A, Ruggiero AM, Lund D, Wright J, et al. Tachycardia, reduced vagal capacity, and age-dependent ventricular dysfunction arising from diminished expression of the presynaptic choline transporter. Am J Physiol Heart Circ Physiol. 2010;299:H799-810.

[15] Kent KM, Epstein SE, Cooper T, Jacobowitz DM. Cholinergic innervation of the canine and human ventricular conducting system. Anatomic and electrophysiologic correlations. Circulation. 1974;50:948-55.

[16] Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc Res. 2004;62:112-21.

[17] Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, Gomes ER, et al. Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. Journal of molecular and cellular cardiology. 2012.

[18] Rana OR, Schauerte P, Kluttig R, Schroder JW, Koenen RR, Weber C, et al. Acetylcholine as an age-dependent non-neuronal source in the heart. Auton Neurosci. 2010;156:82-9.

[19] Kakinuma Y, Akiyama T, Sato T. Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J. 2009;276:5111-25.

[20] Kakinuma Y, Tsuda M, Okazaki K, Akiyama T, Arikawa M, Noguchi T, et al. Heart-specific overexpression of choline acetyltransferase gene protects murine heart against ischemia through hypoxia-inducible factor-1alpha-related defense mechanisms. Journal of the American Heart Association. 2013;2:e004887.

[21] Roy A, Fields WC, Rocha-Resende C, Resende RR, Guatimosim S, Prado VF, et al. Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013.

[22] Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. The Journal of clinical investigation. 2010;120:408-21.

[23] de Castro BM, De Jaeger X, Martins-Silva C, Lima RD, Amaral E, Menezes C, et al. The vesicular acetylcholine transporter is required for neuromuscular development and function. Mol Cell Biol. 2009;29:5238-50.

[24] Prado VF, Martins-Silva C, de Castro BM, Lima RF, Barros DM, Amaral E, et al. Mice deficient for the vesicular acetylcholine transporter are myasthenic and have deficits in object and social recognition. Neuron. 2006;51:601-12.

[25] Kolisnyk B, Guzman MS, Raulic S, Fan J, Magalhaes AC, Feng G, et al. ChAT-ChR2-EYFP mice have enhanced motor endurance but show deficits in attention and several additional cognitive domains. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2013;33:10427-38.

[26] Nagy PM, Aubert I. Overexpression of the vesicular acetylcholine transporter increased acetylcholine release in the hippocampus. Neuroscience. 2012;218:1-11.

[27] Roy A, Fields WC, Rocha-Resende C, Resende RR, Guatimosim S, Prado VF, et al. Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013;27:5072-82.

[28] Crittenden JR, Lacey CJ, Lee T, Bowden HA, Graybiel AM. Severe drug-induced repetitive behaviors and striatal overexpression of VAChT in ChAT-ChR2-EYFP BAC transgenic mice. Frontiers in neural circuits. 2014;8:57.

[29] Zhao S, Ting JT, Atallah HE, Qiu L, Tan J, Gloss B, et al. Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function. Nat Methods. 2011;8:745-52.

[30] Roy A, Lara A, Guimaraes D, Pires R, Gomes ER, Carter DE, et al. An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with decreased cholinergic neurotransmission. PloS one. 2012;7:e39997.

[31] Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, de Morais Gomes ER, et al. Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. Journal of molecular and cellular cardiology. 2012;53:206-16.

[32] Lau JK, Brown KC, Thornhill BA, Crabtree CM, Dom AM, Witte TR, et al. Inhibition of cholinergic signaling causes apoptosis in human bronchioalveolar carcinoma. Cancer research. 2013;73:1328-39.

[33] Beraldo FH, Soares IN, Goncalves DF, Fan J, Thomas AA, Santos TG, et al. Stress-inducible phosphoprotein 1 has unique cochaperone activity during development and regulates cellular response to ischemia via the prion protein. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2013.

[34] Bravo DT, Kolmakova NG, Parsons SM. Choline is transported by vesicular acetylcholine transporter. Journal of neurochemistry. 2004;91:766-8.

[35] Bravo DT, Kolmakova NG, Parsons SM. New transport assay demonstrates vesicular acetylcholine transporter has many alternative substrates. Neurochemistry international. 2005;47:243-7.

[36] Sugaya T, Nishimatsu S, Tanimoto K, Takimoto E, Yamagishi T, Imamura K, et al. Angiotensin II type 1a receptor-deficient mice with hypotension and hyperreninemia. The Journal of biological chemistry. 1995;270:18719-22.

[37] Oliverio MI, Best CF, Kim HS, Arendshorst WJ, Smithies O, Coffman TM. Angiotensin II responses in AT1A receptor-deficient mice: a role for AT1B receptors in blood pressure regulation. The American journal of physiology. 1997;272:F515-20.

[38] Sadoshima J, Izumo S. Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. Circulation research. 1993;73:413-23.

[39] Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. Cell. 1993;75:977-84.

[40] Shanmugam P, Valente AJ, Prabhu SD, Venkatesan B, Yoshida T, Delafontaine P, et al. Angiotensin-II type 1 receptor and NOX2 mediate TCF/LEF and CREB dependent WISP1 induction and cardiomyocyte hypertrophy. Journal of molecular and cellular cardiology. 2011;50:928-38.

[41] Goldenberg I, Grossman E, Jacobson KA, Shneyvays V, Shainberg A. Angiotensin II-induced apoptosis in rat cardiomyocyte culture: a possible role of AT1 and AT2 receptors. Journal of hypertension. 2001;19:1681-9.

[42] Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA, Harrison DG. Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. Circulation. 1997;95:588-93.

[43] Nishiyama A, Fukui T, Fujisawa Y, Rahman M, Tian RX, Kimura S, et al. Systemic and Regional Hemodynamic Responses to Tempol in Angiotensin II-Infused Hypertensive Rats. Hypertension. 2001;37:77-83.

[44] Ren J, Qin C, Hu F, Tan J, Qiu L, Zhao S, et al. Habenula "cholinergic" neurons corelease glutamate and acetylcholine and activate postsynaptic neurons via distinct transmission modes. Neuron. 2011;69:445-52.

[45] Li M, Zheng C, Sato T, Kawada T, Sugimachi M, Sunagawa K. Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats. Circulation. 2004;109:120-4.

[46] Zhang Y, Popovic ZB, Bibevski S, Fakhry I, Sica DA, Van Wagoner DR, et al. Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model. Circ Heart Fail. 2009;2:692-9.

[47] Handa T, Katare RG, Kakinuma Y, Arikawa M, Ando M, Sasaguri S, et al. Anti-Alzheimer's drug, donepezil, markedly improves long-term survival after chronic heart failure in mice. J Card Fail. 2009;15:805-11.

[48] Li M, Zheng C, Kawada T, Inagaki M, Uemura K, Shishido T, et al. Donepezil markedly improves long-term survival in rats with chronic heart failure after extensive myocardial infarction. Circ J. 2013;77:2519-25.

[49] Lataro RM, Silva CA, Fazan R, Jr., Rossi MA, Prado CM, Godinho RO, et al. Increase in parasympathetic tone by pyridostigmine prevents ventricular dysfunction during the onset of heart failure. American journal of physiology Regulatory, integrative and comparative physiology. 2013;305:R908-16.

[50] Schwartz PJ, De Ferrari GM, Sanzo A, Landolina M, Rordorf R, Raineri C, et al. Long term vagal stimulation in patients with advanced heart failure: first experience in man. Eur J Heart Fail. 2008;10:884-91.

[51] Nordstrom P, Religa D, Wimo A, Winblad B, Eriksdotter M. The use of cholinesterase inhibitors and the risk of myocardial infarction and death: a nationwide cohort study in subjects with Alzheimer's disease. European heart journal. 2013;34:2585-91.

[52] Kakinuma Y, Akiyama T, Okazaki K, Arikawa M, Noguchi T, Sato T. A non-neuronal cardiac cholinergic system plays a protective role in myocardium salvage during ischemic insults. PloS one. 2012;7:e50761.

Chapter 6

SUMMARY AND CONCLUSIONS

6 Chapter 6

6.1 Summary of Major Findings

A decrease in vagal-mediated cholinergic signaling is observed in the failing myocardium; however, the exact role of cholinergic signaling in the regulation of cardiac function was unclear. In this thesis, we sought to determine whether alterations in neuronal and/or non-neuronal cholinergic signaling leads to changes in cardiac homeostasis, in particular inotropic and chronotropic responses as well as transcriptional regulation. Furthermore, our goal was to elucidate whether ACh signaling plays a role in delaying the progression of cardiac remodeling under pathological conditions.

Chapter 2 of this thesis was designed to identify molecular alterations associated with deficient systemic cholinergic signaling. The mice utilized in Chapter 2 presents a global reduction of approximately 70% in levels of the vesicular acetylcholine transporter (VAChT). Our laboratory previously reported that these mice display cardiac dysfunction and a phenotype similar to heart failure [1]. Importantly, many of the functional and molecular changes associated with reduced VAChT expression (VAChT KD^{HOM} mice) could be attenuated with the use of pyridostigmine, a peripheral cholinesterase inhibitor [1]. These data suggested that lack of ACh secretion was, in fact, the driving force that led to changes in ventricular function. As such, we further characterized the molecular alterations in these mice due to deficient cholinergic signaling. A microarray analysis was performed using cardiac tissue and we identified numerous transcriptional changes, including changes in genes that regulate fatty acid synthesis. Furthermore, we identified an increase in reactive oxygen species (ROS) in cardiomyocytes from mice with decreased ACh secretion, which may partially be due to the transcriptional changes observed here. Finally, we utilized another model of autonomic dysfunction with enhanced adrenergic signaling and observed similar transcriptional changes as those observed with cholinergic deficient mice. The data presented here further implicate autonomic imbalance as a main contributor to cardiac dysfunction.

As previously mentioned, parasympathetic innervation of the ventricles is very sparse, where it is mainly restricted to the cardiac conduction system [2-4]. Interestingly, previous studies suggested that ventricular cardiomyocytes also express prototypical markers of the cholinergic system, which may act to regulate ventricular function [5, 6]. In Chapter 3, we discovered that murine cardiomyocytes posses the machinery to produce and secrete ACh. Furthermore, cardiomyocyte-derived ACh was able to prevent hypertrophy and molecular remodeling in cardiomyocytes in response to hyperadrenergic stimulation, *in vitro*. Specifically, preserving extracellular ACh levels through treatment with cholinesterase inhibitors led to decreased cardiomyocyte hypertrophy in response to isoproterenol treatment, reduced expression of cardiac stress markers, decreased NFAT nuclear translocation and decreased peak systolic calcium as compared to isoproterenol treatment alone. Together, these data provide support to the concept that ACh secreted from cardiomyocytes may act in an autocrine/paracrine fashion to offset the deleterious effects of enhanced sympathetic signaling in cardiac disease.

In Chapter 4, we determined whether this non-neuronal system identified in isolated and cultured cardiomyocytes plays a role in regulating cardiac function. We observed that the cardiac non-neuronal cholinergic system (NNCS) is critical in regulating heart activity and remodeling, *in vivo*. In fact, inhibition of ACh secretion from cardiomyocytes through selective deletion of VAChT resulted in delayed heart rate recovery following physiological stress, including exercise, as well as significant ventricular remodeling. Under basal conditions, cardiomyocytes lacking the VAChT displayed hypertrophy and molecular remodeling including activation of the fetal gene program, increased reactive oxygen species (ROS) production and enhanced peak systolic calcium. The molecular remodeling observed in these mice resulted in abnormal inotropic responses following a bolus dose of isoproterenol, which suggests that cardiomyocyte-derived ACh contributes to the regulation of cardiac function under stress. These data support the notion that the cardiac NNCS is critical in regulating physiological responses, likely by amplifying cholinergic signaling in the heart. Our experiments also point to the mechanisms by which ACh is secreted from cardiomyocytes. It has previously been shown that ACh release from other cell types, including epithelial cells, is dependent on organic cation

transporters (OCT) rather than VAChT [7, 8]. In cardiomyocytes, we observed that both pharmacological and genetic inhibition of VAChT prevent ACh secretion thus verifying this transporter as an essential mediator of ACh release from cardiac myocytes.

Importantly, the VAChT is part of the major facilitator transporter superfamily, and can transport other organic substrates, which can have distinct physiological effects [9, 10]. As such, mice with cardiomyocyte-specific deletion of ChAT (cChAT mice) were utilized in Chapters 4 and 5 to confirm that the altered cardiac function observed in cVAChT mice was due to altered cholinergic signaling. Many of the remodeling processes and altered heart rate regulation observed in cVAChT mice were also present in cChAT mice. These data provided definitive evidence supporting the notion that the observed alterations in cardiac function are due to inhibition of cardiomyocyte-specific ACh production or release.

Finally, in Chapter 5, we identified that this intrinsic cholinergic system in the heart is also present in human cardiomyocytes. To the best of our knowledge, this is the first documentation of the presence of cholinergic machinery in human cardiomyocytes, which also have conserved expression of prototypic markers of the cholinergic system. Importantly, this system might be of functional significance in cardiac disease as failing human cardiomyocytes exhibit increased VAChT expression, which likely leads to an increase in ACh secretion directly from cardiomyocytes. Importantly, previous data suggest that parasympathetic tone is downregulated in HF, which contributes to autonomic dysfunction. However, previous experiments suggest that cardiac sympathetic nerves transdifferentiate into cholinergic neurons thereby increasing ACh secretion in heart failure, which leads to decreased mortality [11]. In addition to this previous study, our data suggest that ventricular ACh secretion may also be increased in human heart failure. Together, these data indicate that, although compensatory mechanisms may be activated in HF in order to increase cholinergic signaling, this is still not enough to completely abolish the ventricular remodeling observed in cardiac disease. Furthermore, it hints at the possibility that inhibition of ACh secretion may lead to worse prognosis and outcomes in heart failure.

Given that VAChT expression was increased in diseased myocardium, we examined whether a systemic increase in VAChT expression and ACh release leads to altered cardiac function and adverse effects under physiological conditions. Although our data suggest that VAChT is not overexpressed in ventricular cardiomyocytes in *ChAT-ChR2-EYFP* mice, we sought to determine whether increased cholinergic signaling in the atria could alter cardiac function. VAChT overexpression and increased cardiac atrial ACh secretion did not lead to any significant inotropic or chronotropic differences. These data suggest that the increase in VAChT expression observed in failing human myocardium likely plays a protective role rather than contribute to the adverse phenotypes present in heart failure. In order to further determine the role of the NNCS in the onset and progression of cardiac disease, we utilized cVAChT mice, which were characterized in Chapter 4. Chronic angiotensin II (Ang II) treatment led to enhanced cardiac remodeling and ventricular dysfunction in mice lacking the non-neuronal cholinergic system. Specifically, cVAChT mice displayed greater defects in ventricular fractional shortening and ejection fraction following Ang II treatment. Furthermore, pathological responses were also increased in cVAChT mice, as greater disruption of myocardial structure and increased fibrotic response were apparent in conditional VAChT knockout mice. These data provide further support to the notion that the non-neuronal cholinergic system is crucial in reducing the progression of ventricular remodeling.

6.2 Limitations and Future Studies

The VAChT KD^{HOM} mice utilized in Chapter 2 display a global decrease in VAChT expression and, therefore, ACh release. This results in a decrease in both parasympathetic and sympathetic signaling, due to the fact that ACh mediates pre-ganglionic sympathetic signaling. Moreover, these mice also show a deficit in cardiomyocyte-derived ACh signaling (Chapter 3). It is possible that the transcriptional alterations observed in cardiac tissue from VAChT KD^{HOM} mice are due to abnormal sympathetic, parasympathetic

and/or non-neuronal signaling. However, this mouse line did not allow us to elucidate the implications of exclusively altering parasympathetic signaling.

Furthermore, decreased systemic cholinergic signaling does not solely lead to decreased ACh secretion at the level of the heart. Rather, there is significant data suggesting that vagal signaling can lead to activation of the cholinergic anti-inflammatory pathway, which can activate α7 nicotinic acetylcholine receptors (α7nAChRs) on macrophages and inhibit pro-inflammatory cytokine release from these cells, an effect that can be blunted using selective nicotinic antagonists or α7nAChR knockout mice [12-16]. It is likely that some of the ventricular dysfunction and pathological responses observed in the VAChT KD^{HOM} mice is due to defective regulation of this inflammatory response rather than direct effects of ACh in the myocardium. In support of this notion, there is evidence that this mouse line presents abnormal anti-inflammatory responses [17]. As such, future studies will need to be designed to analyze the specific importance of this cholinergic anti-inflammatory pathway in the regulation of heart disease. To this effect, ventricular function and pathological remodeling will be analyzed in α7nAChR KO mice via either left anterior descending coronary artery (LAD) ligation or Ang II infusion. A detailed analysis of cardiac remodeling in two distinct models of cardiac disease will determine whether this pathway has a significant impact on progression of heart disease. Furthermore, it will provide vital information regarding the contribution of the cholinergic anti-inflammatory pathway in mediating the positive effects of vagal stimulation.

In Chapter 3, we observed that cardiomyocytes are able to secrete ACh and this intrinsic cholinergic system may participate in the normal response to adrenergic stimulation. Although our data suggest that the NNCS has an important physiological function, the mechanisms that trigger ACh release from cardiomyocytes have yet to be elucidated. VAChT appears to be present in exocytic vesicles, which are maintained in a pool at the perinuclear region in cardiomyocytes [18]. It is possible that these exocytic vesicles with ACh are retained near the nucleus to prevent inadvertent exocytosis of ACh due to calcium influx during systolic contraction. However, no detailed studies have yet been

conducted in order to truly explore the mechanisms responsible for triggering ACh release from cardiomyocytes.

Although this concept has yet to be tested, it is possible that ACh secretion is dependent on sympathetic signaling, which leads to exocytosis of ACh-containing vesicles. This notion is supported by the data presented in Chapter 4 which suggest that inhibition of cardiomyocyte-derived ACh, *in vivo,* leads to delayed heart rate recovery following exercise. Increased parasympathetic signaling has long been implicated in heart rate recovery following physiological stress; however, our data suggest that increased sympathetic signaling may induce secretion of cardiac ACh, which can act to amplify parasympathetic signaling and enhance heart rate recovery. In order to test the hypothesis that sympathetic signaling can mediate ACh release from cardiomyocytes, further *in vitro* studies will need to be conducted. Cultured neonatal cardiomyocytes can be subjected to acute treatment with the β-adrenergic agonist, isoproterenol, and extracellular levels of ACh can be analyzed.

One of the major changes we identified in Chapter 4 involved significant ventricular hypertrophy and remodeling due to lack of the non-neuronal cholinergic system in mice. However, the exact mechanisms that lead to ventricular dysfunction and molecular remodeling are still unclear. One possibility is that the enhanced heart rate response observed in these animals is due to increased SAN activity, which results in a pacing-induced pathogenesis model of HF. Under these circumstances, increased chronotropic responses may be expected to place greater strain on cardiac muscle and activate pathways leading to ventricular remodeling. Conversely, it is possible that cardiomyocyte-derived ACh plays a direct role in regulating the function of ventricular cardiomyocytes and, in the absence of this NNCS system, cardiomyocytes are susceptible to molecular remodeling. The latter possibility is in agreement with previous work from another group suggesting that treatment with the cholinesterase inhibitor, donepezil, preserves cardiomyocyte-secreted ACh and activates ChAT transcription, thereby increasing ACh production in cardiomyocytes [6]. Furthermore, the authors suggested that increasing ACh signaling can decrease oxygen consumption and energy metabolism,

which may serve to preserve myocyte function [6]. In order to determine which of these possibilities leads to the observed phenotype, cVAChT mice must be chronically treated with the peripheral cholinesterase inhibitor, pyridostigmine (PYR). In the event that cVAChT mice exhibit pacing-induced ventricular dysfunction, treatment with PYR may be able to reverse many of the molecular changes observed in these animals, as seen in previously published data from our group [1]. However, if the intrinsic cholinergic system in cardiomyocytes has a direct protective role in the ventricles via autocrine/paracrine signaling, PYR treatment will produce no positive outcomes due to the fact that cardiac myocytes lack the ability to secrete ACh.

Irrespective of the specific pathways that lead to the ventricular remodeling observed in Chapter 4, it is evident that cardiomyocytes from hearts lacking this intrinsic cholinergic system display baseline molecular remodeling. One potential mechanism for the observed cardiomyocyte remodeling that has been explored in this thesis involves nuclear translocation of GRK5, which has been implicated in cardiac hypertrophy [19]. However, there are likely other mechanisms through which ACh is able to regulate several different remodeling pathways and downstream functional effects that have not been fully elucidated in this thesis.

One of these pathways involves changes in calcium signaling in isolated cardiomyocytes from cVAChT mice. A significant increase in peak systolic calcium levels is observed in isolated cardiomyocytes, which often results in increased transport of Ca^{2+} into the mitochondria where it can lead to mitochondrial Ca^{2+} overload and increased ROS production [20]. It is well known that an increase in mitochondrial ROS levels leads to uncoupling of oxidative phosphorylation, which leads to a decline in ATP production, as well as cell death [21-23]. Furthermore, this decrease in ATP production, coupled with the loss of phosphocreatine (PCr), is often seen in cardiac disease and the PCr/ATP ratio appears to be a strong predictor of mortality [24-27]. Together, these data suggest the need to determine whether the increased mitochondrial ROS production in cVAChT myocytes contribute to the observed remodeling and ventricular dysfunction. An *in vitro* primary culture model with cardiomyocytes will be necessary to dissect the specific

mechanisms regulating mitochondrial Ca^{2+} overload and ROS production and will allow us to further determine the exact pathways regulated by the cardiac intrinsic cholinergic system.

In addition, decreased energy production will be an interesting avenue to explore in cVAChT mice. Both ATP levels as well as cardiac energetic reserves (PCr) are critical to the maintenance of normal cardiac function. Furthermore, the transcriptional changes observed in another cholinergic deficient mouse line (VAChT KD^{HOM}) in Chapter 2 suggest that cardiac energetics are regulated by cholinergic signaling. Therefore, future studies should seek to determine whether decreased ATP production can serve as a potential mediator of cardiac dysregulation in cVAChT mice.

Throughout our studies, we have shown that cardiomyocytes possess the machinery to secrete ACh, which has significant functional effects. However, it is still unclear whether other cell types in the heart are also able to secrete ACh and regulate cardiac physiology through autocrine/paracrine signaling. It is not improbable that other cell types in the myocardium are capable of producing extraneuronal ACh. In fact, it has already been reported that several different cell types generate *de novo* ACh, including keratinocytes [28], epithelial cells [29, 30] as well as pancreatic α-cells where non-neuronal ACh primes β-cells to secrete insulin in humans [31]. It will be important to determine whether different cell types in the heart possess the machinery to produce ACh and whether altered ACh secretion from these cells is functionally significant. Cardiac fibroblasts, in particular, would be a good target as they have been implicated in the adverse myocardial remodeling that occurs in heart disease [32]. Future studies will involve isolation of cardiac fibroblasts and analysis of response to external stressors, including inotropic agents. If these *in vitro* studies prove promising, it would be necessary to investigate whether cardiac fibroblasts play a role under both physiological and especially pathological conditions, *in vivo*.

In addition to the possibility that other cell types in the heart can secrete ACh, it is also likely that ACh secretion from cardiomyocytes can modulate ventricular function by

regulating the actions of other cardiac cell types via paracrine signaling. Although this possibility has never been explored, it may provide a novel pathway through which cardiomyocyte-derived ACh has its functional effects, *in vivo*. Endothelial cells, which are abundantly present in the myocardium, are one particular cell type that may directly be affected by cardiac non-neuronal ACh. It's possible that extraneuronal ACh may bind to M_3 receptors on endothelial cells and induce vasodilation of vascular smooth muscle cells in ventricular coronary arteries. Although it is technically difficult to determine whether cardiomyocyte-secreted ACh acts through paracrine signaling in other cell types, the possibility is certainly enticing. It is important to note that the NNCS is not unique to the cell types mentioned above. In fact, the NNCS plays an active and important role in regulating immune function because T-cells are able to secrete ACh, which can then act in an autocrine/paracrine fashion to inhibit inflammatory cytokine production [33, 34].

In Chapter 5, we show that a systemic increase in VAChT expression does not lead to any adverse cardiovascular phenotypes. Rather, the mice are relatively normal and also appear to exhibit greater heart rate regulation in response to physiological stress. The availability of animal models makes it possible to test the notion that increased ACh secretion from T cells can enhance cholinergic anti-inflammatory signaling and play a protective role under pathological states. The mouse line we utilized in Chapter 5, *ChAT-ChR2-EYFP*, overexpresses VAChT in all tissues where ChAT is expressed, including T cells. Future studies will look to determine whether increased secretion of this T cell non-neuronal ACh, in conjunction with enhanced cardiac cholinergic signaling, can regulate the onset and progression of ventricular dysfunction in mice following induction of a pathological response. Furthermore, since VAChT expression appears to be increased in human failing cardiomyocytes, it will be interesting to determine whether HF also correlates with increased ACh production and signaling in the cholinergic anti-inflammatory pathway.

6.3 Significance of Research and Conclusion

Although the exact role of the parasympathetic nervous system in cardiac dysfunction and heart failure has yet to be completely understood, the data presented in this thesis highlights the importance of modulating this system in heart disease. Previous data have made it clear that increasing ACh levels, through either vagal stimulation or cholinesterase inhibitors, can act through a variety of avenues and reduce cardiac remodeling in several different models of heart disease. In addition, increasing non-neuronal expression of ChAT specifically in cardiomyocytes has been shown to attenuate ventricular remodeling. The data presented in this thesis furthers our understanding regarding the regulation of cardiac function by both neuronal and non-neuronal cholinergic signaling. Notably, we show that this system modulates cardiac activity not only under pathological conditions but also in the absence of an external stressor. As such, the cholinergic system may serve as an important pharmacological target in patients with heart disease.

Recent efforts to repurpose drugs in cancer and neurological diseases are underway with the leadership of the NIH [35]. The safety and tolerance of cholinesterase inhibitors are well known, due to their wide use in Alzheimer's disease. Therefore, it would be easy to repurpose this class of drugs for heart disease. Modulation of cholinergic signaling may serve as a novel, unexplored clinical avenue for the treatment of HF in humans. However, clinical data on the benefits of cholinesterase inhibitors are still scant. Clinical trials are certainly warranted in order to test potential benefits of adding cholinesterase inhibitors to current therapies and evaluate the long-term effects of cholinesterase inhibitors at different stages of cardiac disease and heart failure.

6.4 References

[1] Lara A, Damasceno DD, Pires R, Gros R, Gomes ER, Gavioli M, et al. Dysautonomia due to reduced cholinergic neurotransmission causes cardiac remodeling and heart failure. Mol Cell Biol. 2010;30:1746-56.

[2] Kent KM, Epstein SE, Cooper T, Jacobowitz DM. Cholinergic innervation of the canine and human ventricular conducting system. Anatomic and electrophysiologic correlations. Circulation. 1974;50:948-55.

[3] Hoover DB, Ganote CE, Ferguson SM, Blakely RD, Parsons RL. Localization of cholinergic innervation in guinea pig heart by immunohistochemistry for high-affinity choline transporters. Cardiovasc Res. 2004;62:112-21.

[4] Crick SJ, Wharton J, Sheppard MN, Royston D, Yacoub MH, Anderson RH, et al. Innervation of the human cardiac conduction system. A quantitative immunohistochemical and histochemical study. Circulation. 1994;89:1697-708.

[5] Rana OR, Schauerte P, Kluttig R, Schroder JW, Koenen RR, Weber C, et al. Acetylcholine as an age-dependent non-neuronal source in the heart. Auton Neurosci. 2010;156:82-9.

[6] Kakinuma Y, Akiyama T, Sato T. Cholinoceptive and cholinergic properties of cardiomyocytes involving an amplification mechanism for vagal efferent effects in sparsely innervated ventricular myocardium. FEBS J. 2009;276:5111-25.

[7] Lips KS, Volk C, Schmitt BM, Pfeil U, Arndt P, Miska D, et al. Polyspecific cation transporters mediate luminal release of acetylcholine from bronchial epithelium. Am J Respir Cell Mol Biol. 2005;33:79-88.

[8] Kummer W, Wiegand S, Akinci S, Wessler I, Schinkel AH, Wess J, et al. Role of acetylcholine and polyspecific cation transporters in serotonin-induced bronchoconstriction in the mouse. RespirRes. 2006;7:65.

[9] Bravo DT, Kolmakova NG, Parsons SM. Choline is transported by vesicular acetylcholine transporter. Journal of neurochemistry. 2004;91:766-8.

[10] Bravo DT, Kolmakova NG, Parsons SM. New transport assay demonstrates vesicular acetylcholine transporter has many alternative substrates. Neurochemistry international. 2005;47:243-7.

[11] Kanazawa H, Ieda M, Kimura K, Arai T, Kawaguchi-Manabe H, Matsuhashi T, et al. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. The Journal of clinical investigation. 2010;120:408-21.

[12] Tracey KJ. The inflammatory reflex. Nature. 2002;420:853-9.

[13] Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature. 2003;421:384-8.

[14] Guarini S, Altavilla D, Cainazzo MM, Giuliani D, Bigiani A, Marini H, et al. Efferent vagal fibre stimulation blunts nuclear factor-kappaB activation and protects against hypovolemic hemorrhagic shock. Circulation. 2003;107:1189-94.

[15] Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J, et al. Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem. 2004;89:337-43.

[16] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000;405:458-62.

[17] Machado MP, Rocha AM, de Oliveira LF, de Cuba MB, de Oliveira Loss I, Castellano LR, et al. Autonomic nervous system modulation affects the inflammatory immune response in mice with acute Chagas disease. Experimental physiology. 2012;97:1186-202.

[18] Ferlito M, Fulton WB, Zauher MA, Marban E, Steenbergen C, Lowenstein CJ. VAMP-1, VAMP-2, and syntaxin-4 regulate ANP release from cardiac myocytes. Journal of molecular and cellular cardiology. 2010;49:791-800.

[19] Martini JS, Raake P, Vinge LE, DeGeorge BR, Jr., Chuprun JK, Harris DM, et al. Uncovering G protein-coupled receptor kinase-5 as a histone deacetylase kinase in the nucleus of cardiomyocytes. Proceedings of the National Academy of Sciences of the United States of America. 2008;105:12457-62.

[20] Gunter TE, Buntinas L, Sparagna G, Eliseev R, Gunter K. Mitochondrial calcium transport: mechanisms and functions. Cell calcium. 2000;28:285-96.

[21] Halestrap AP. Calcium, mitochondria and reperfusion injury: a pore way to die. Biochemical Society transactions. 2006;34:232-7.

[22] Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res. 2004;61:372-85.

[23] Crompton M, Virji S, Doyle V, Johnson N, Ward JM. The mitochondrial permeability transition pore. Biochemical Society symposium. 1999;66:167-79.

[24] Nakae I, Mitsunami K, Omura T, Yabe T, Tsutamoto T, Matsuo S, et al. Proton magnetic resonance spectroscopy can detect creatine depletion associated with the progression of heart failure in cardiomyopathy. J Am Coll Cardiol. 2003;42:1587-93.

[25] Shen W, Asai K, Uechi M, Mathier MA, Shannon RP, Vatner SF, et al. Progressive loss of myocardial ATP due to a loss of total purines during the development of heart failure in dogs: a compensatory role for the parallel loss of creatine. Circulation. 1999;100:2113-8.

[26] Neubauer S, Horn M, Cramer M, Harre K, Newell JB, Peters W, et al. Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. Circulation. 1997;96:2190-6.

[27] Hansch A, Rzanny R, Heyne JP, Leder U, Reichenbach JR, Kaiser WA. Noninvasive measurements of cardiac high-energy phosphate metabolites in dilated cardiomyopathy by using 31P spectroscopic chemical shift imaging. Eur Radiol. 2005;15:319-23.

[28] Grando SA, Kist DA, Qi M, Dahl MV. Human keratinocytes synthesize, secrete, and degrade acetylcholine. The Journal of investigative dermatology. 1993;101:32-6.

[29] Reinheimer T, Munch M, Bittinger F, Racke K, Kirkpatrick CJ, Wessler I. Glucocorticoids mediate reduction of epithelial acetylcholine content in the airways of rats and humans. European journal of pharmacology. 1998;349:277-84.

[30] Nguyen VT, Hall LL, Gallacher G, Ndoye A, Jolkovsky DL, Webber RJ, et al. Choline acetyltransferase, acetylcholinesterase, and nicotinic acetylcholine receptors of human gingival and esophageal epithelia. Journal of dental research. 2000;79:939-49.

[31] Rodriguez-Diaz R, Dando R, Jacques-Silva MC, Fachado A, Molina J, Abdulreda MH, et al. Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans. Nature medicine. 2011;17:888-92.

[32] Porter KE, Turner NA. Cardiac fibroblasts: at the heart of myocardial remodeling. Pharmacology & therapeutics. 2009;123:255-78.

[33] Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science. 2011;334:98-101.

[34] Fujii T, Tsuchiya T, Yamada S, Fujimoto K, Suzuki T, Kasahara T, et al. Localization and synthesis of acetylcholine in human leukemic T cell lines. J Neurosci Res. 1996;44:66-72.

[35] Collins FS. Mining for therapeutic gold. Nature reviews Drug discovery. 2011;10:397.

Curriculum Vitae

Name: Ashbeel Roy

Post-secondary Education and Degrees:

The University of Western Ontario
London, Ontario, Canada
2005-2009 B.Sc.

The University of Western Ontario
London, Ontario, Canada
2009-2014 Ph.D.

Honours and Awards:

Western Graduate Research Scholarship
UWO, London, Canada
2009-2014

Queen Elizabeth II Graduate Scholarship
2011-2012

Poster Award – London Health Research Day
London, Canada
Mar. 2012

Oral Presentation Award – Canadian Hypertension Congress
Toronto, Canada
Oct. 2012

Schulich Graduate Thesis Research Award
London, Canada
2012-2013

Province of Ontario Graduate Scholarship
2012-2013, 2013-2014

Cobban Award for Heart and Stroke Research
London, Canada
May, 2013

Oral Presentation Award – Canadian Hypertension Congress
Montreal, Canada
Oct. 2013

Poster Award - Charles W. Gowdey Research Day
UWO, London, Canada
Nov. 2013

Mogensen Research Scholarship
UWO, London, Canada
Nov. 2013

Related Work Experience

Teaching Assistant
The University of Western Ontario
2012-2014

Publications:

Roy A, Fields WC, Rocha-Resende C, Resende RR, Guatimosim S, Prado VF, Gros R, Prado MA. (2013). Cardiomyocyte-secreted acetylcholine is required for maintenance of homeostasis in the heart. *FASEB J.* 2013 Dec;27(12):5072-82.

Prado VF, Roy A, Kolisnyk B, Gros R, Prado MA. (2013). Regulation of cholinergic activity by the vesicular acetylcholine transporter. *Biochemical Journal.* 2013 Mar 1;450(2):265-74.

Roy A, Lara A, Guimarães D, Pires R, Gomes ER, Carter DE, Gomez MV, Guatimosim S, Prado VF, Prado MA, Gros R. (2012). An analysis of the myocardial transcriptome in a mouse model of cardiac dysfunction with decreased cholinergic neurotransmission. *PLoS One.* 2012;7(6):e39997.

Rocha-Resende C, Roy A, Resende R, Ladeira MS, Lara A, de Morais Gomes ER, Prado VF, Gros R, Guatimosim C, Prado MA, Guatimosim S. (2012). Non-neuronal cholinergic machinery present in cardiomyocytes offsets hypertrophic signals. *Journal of Molecular and Cellular Cardiology.* 2012 Aug;53(2):206-16.